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SOME MORPHOLOGICAL AND BIOLOGICAL CHARACTERS OF THE SPIRILLA (VIBRIO FETUS, N. SP.) ASSOCIATED WITH DISEASE OF THE FETAL MEMBRANES IN CATTLE.

By THEOBALD SMITH, M.D., and MARIAN S. TAYLOR.

(*From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.*)

Plate 16.

(Received for publication, June 3, 1919.)

In an earlier communication[1] a spirillum of definite morphological and cultural characters was described as being associated in a series of cases with what is commonly known as infectious abortion in cattle. In the following pages the statements there made concerning the biology of the spirillum are amplified and supplemented by fresh observations and by studies on the agglutinative affinities of the various strains. In all, 24 fetal strains have been kept under cultivation. Of these one (No. 308) is a slightly modified, aberrant type. All were obtained from one large herd into which cattle from outside are introduced at irregular intervals.

Morphological Characters.

In films and in hanging drop preparations from fetal fluids and cultures therefrom, the spirilla appear as fine wavy or sinuous lines of various lengths. The smallest forms appear as minute curved S-shaped lines; the longest may stretch nearly across the field of the microscope. If we assume that the spirillum is in the form of a spiral or corkscrew, the diameter of the spiral is small. The spiral is drawn out, as it were, becoming in some cultures almost a straight line. In dried and stained films the spiral becomes a shallow sinuous line (Fig. 7). No segmentations are distinguishable in the longer forms.

[1] Smith, T., *J. Exp. Med.*, 1918, xxviii, 701.

As regards size, the width of the spirillum stained in alkaline methylene blue is probably not over 0.2 to 0.3μ, the shortest form about 1.5 to 2μ long. A common size in the fetal fluids consists of about two complete turns and measures 4 to 5μ in length. As stated above, the diameter of the spiral or turn varies somewhat, but it averages about 0.5μ.

The organism stains fairly well in alkaline methylene blue provided the staining is prolonged, preferably over night. It stains much more deeply in diluted aniline water gentian violet (Fig. 6), but the former stain is to be preferred since it requires no decoloration, a process likely to decolorize the spirillum itself.

When Löffler's flagellar stain, as modified by Moore,[2] is applied, the organism is much thicker, as shown in Figs. 1 to 5. A flagellum is found attached to one or both poles of the spirillum. Actual counts of the spirilla of eight different strains in a given number of fields indicated that organisms having unipolar flagella are far more numerous than those with bipolar flagella. The latter may be considered relatively scarce. The unipolar forms may be individuals recently set free by division, the bipolar forms ready to divide. Longer spirilla have been seen with several short lateral flagella in addition to the terminal ones. This appearance may be due to forms which have divided but not yet separated, the flagella of the daughter cells having been formed in the meantime. The character of the flagella is best seen in the figures. They are very fine hair-like processes in large undulations or waves, of uniform width throughout.

In hanging drop preparations the shorter forms, from $\frac{1}{2}$ to 2 windings long, are very active. They shoot with lightning rapidity in straight lines across the field in all directions. Only occasionally does one reduce its velocity so that details of the movements become visible. It may then be seen in a few cases that the organism revolves around its longitudinal axis. Whether this motion is used in very rapid propulsion cannot be determined. The longer forms move sluggishly or are quiescent.

Another element brought out by staining and referred to in a former paper[1] is the granules which appear on or within the spirilla. They

[2] Moore, V. A., Wilder quarter century book, Ithaca, 1893, 339.

occur more commonly in old cultures and are probably degeneration forms. Usually the long spirilla contain many, but short forms are not without them. In the latter they are terminal. In the long forms they are arranged along the filament at fairly regular intervals. They stain much more deeply than the rest of the spirillum and suggest the Neisser granules of diphtheria bacilli. The feeble resistance of this spirillum to heat and drying makes any interpretation of these granules as spores improbable.

Owing to the recent extensive additions to our knowledge of spiral organisms as agents of disease and the introduction of new methods of culture, the nomenclature is not in a satisfactory state. Most writers on classification have accepted the generic designation vibrio for short, so called comma forms, and spirillum for distinctly spiral forms. The former have one polar flagellum, rarely more, the latter a tuft of polar flagella. The spiral form here described appears, as a rule, in both long spiral and short comma forms in the fetal fluids and may therefore be regarded as standing intermediate between the comma forms and true spirals. Nevertheless since this organism assumes the comma form in its early, most active stage of multiplication in cultures and has single polar flagella, the generic term vibrio appears most appropriate at the present time. It is therefore designated *Vibrio fetus, n. sp.* The term spirillum we shall continue to use in the text as a general expression for spiral organisms.

Cultural Characters.

In discussing the culture characteristics of *Vibrio fetus* we must distinguish between the earliest and later cultures. The earliest represent the still unchanged animal type, whereas later cultures may become saprophytized. The change from one to the other growth type goes on gradually and no boundary lines in terms of generations or transfers can be definitely assigned.

The earliest growths have been obtained in agar slants containing a small quantity of condensation water or added bouillon. To this a bit of tissue about $\frac{1}{8}$ gm. or an equivalent amount of stomach or other fluid or intestinal contents of the fetus is added and the tubes are sealed with sealing-wax. In such a medium the growth may be

feeble and is likely to be overlooked entirely at first. After 3 or more days of incubation two very narrow grayish white, opaque lines of growth extend upward from beneath the surface of the condensation water where the agar slant abuts against the glass. These lines, less than 1 mm. broad, are at first the only macroscopic indications of growth. Soon an exceedingly thin veil-like layer extends from each of these two lines into the capillary space between agar and glass, often meeting behind and then forming a complete barely visible layer with upper margins variably high and undulating or jagged, but rarely more than 4 cm. above the bottom of the tube. The condensation water is very feebly clouded but motile spirilla are usually found in it. Surface growth occurs only very exceptionally.

The above features persist in subsequent cultures but there are gradually added others. If defibrinated horse or other mammalian blood is added to the condensation water, a layer of growth appears on the surface of the sedimented corpuscles, which grows heavier with the number of transfers. Surface growths appear after months of cultivation either as grayish white films or as isolated, roundish, smooth, glistening colonies, 1 to 2 mm. in diameter.

With the increasing vigor of growth it becomes possible to obtain multiplication on agar slants without the blood and thereafter the strain may be maintained on plain agar in sealed tubes. In the condensation water a viscid whitish sediment made up of spirilla appears. This may be drawn out into threads. This phenomenon seems to point to a kind of mucoid degeneration which is suggested by the unstained sheaths around spirilla in Fig. 8.

In the earliest growths fluid media failed, as a rule, to induce multiplication. This failure may be due to the continually changing relation to oxygen of the different layers of fluids on account of convection currents. The agar medium provides stable conditions different at different points of the slant. The method of cultivation described differs from the methods of other workers in which deep layers of culture media, either solid, semisolid, or fluid, have been used for bacteria sensitive to oxygen. The use of slanted agar in a sealed tube gives better access to the growth than do the deep layers. It provides various degrees of oxygen tension and hence a greater variety of conditions, one of which may fit the organism to be cultivated.

With some strains a fairly vigorous multiplication has occurred in the first cultures, such as growth on the agar surface and membrane formation on the condensation water, festoons and mucoid shreds hanging down into this fluid. This is due to a specially favorable medium, which is mucus from the stomachs of the fetuses transferred to tubes when original cultures are prepared.

After thirty or more transfers with intervals up to a week multiplication has been observed in unsealed agar slants. The reduced oxygen tension in such tubes is obtained, when necessary to the organism, by growth in the capillary space between the mass of agar and the sides of the tube.

When agar was used in deep layers in sealed tubes the growth phenomena corresponded with those of *Bacillus abortus* under like conditions.

The following description applies to saprophytized strains. Fluid agar, thoroughly mixed with the spirillum, was allowed to set. Growth was gradual, appearing after incubation of 2 to 3 days as a fine white line around the circumference of the surface of the agar, and slowly growing down between the agar and the glass as a fine film. Condensation water gathered, became viscid, and growth spread over the surface of the agar. A dark line forming in the agar below the surface was observed, which at the end of a week's incubation resolved itself into a zone of individual colonies. The colonies were small, opaque, and of a yellow color. In some instances the zone appeared directly under the surface and in others it grew from 2 to 5 mm. below the surface. In unsealed tubes there was no condensation water and no growth at the surface, but the subsurface zone appeared heavier than in the sealed cultures.

In agar stabs growth developed slowly, appearing on the 2nd day along the lines of puncture. The final growth along the stab varied from 5 mm. to 3 cm. in length. If a lateral puncture was made, a fine white film gradually spread between the agar and the glass. At the points of inoculation a surface growth developed, and any slight condensation water present became viscid. In unsealed cultures no growth appeared on the surface.

Coagulated and slanted blood serum (horse) did not prove superior to the slanted agar plus a few drops of blood. No liquefaction was noted in the usually feeble growth. Growth was obtained when

defibrinated blood was incorporated with melted agar and the latter slanted. Multiplication did not take place in nutrient gelatin, either at incubator or room temperature. It is not known, therefore, whether a liquefying enzyme exists or not.

After strains had become adapted to plain slanted agar, attempts were made to induce multiplication in fluid media. Growth in simple beef peptone bouillon in sealed and unsealed tubes becomes possible, but the addition of a few drops of defibrinated blood makes multiplication more vigorous. The medium becomes faintly clouded and a slight viscid sediment is formed. A filmy stringy deposit may settle upon the sides if the tubes are slightly inclined and a viscid ring may appear at the surface, but no pellicle. Growth is not improved when paraffin oil covers the fluid column. In milk no recognizable multiplication occurs.

Biological Characters.

Since *Vibrio fetus* has many biological characters in common with *Bacillus abortus*, it would seem that these two widely separated morphological species are drawn nearer together in their physiological characters through adaptation to the same environment on the fetal membranes, causing the same sensitiveness to oxygen tension, the same fastidiousness towards culture media, and indifference to carbohydrates. Marked differences, however, do occur, such as failure on the part of *Vibrio fetus* to multiply on potato, on which *Bacillus abortus* produces a fairly rich pigmented growth.

The length of time that a sealed original culture at room temperature may contain living organisms differs from tube to tube. One culture gave rise to successful subcultures after 139 days, another failed to do so after 31 days. Subcultures on agar plus blood displayed the same differences—one dead after $2\frac{1}{2}$ weeks, another alive after 9 weeks. It is therefore important in maintaining strains to transfer once a week.

When cultures are stored in refrigerators at a temperature of 5–6°C. above freezing their vitality is reduced considerably as compared with that of cultures kept at room temperature.

The reaction of the bouillon may be carried slightly beyond the neutral point of phenolphthalein without interfering with multiplication. Indole is not formed in bouillon. Fermented bouillon with

1 per cent of dextrose, lactose, and saccharose was used in fermentation tubes to determine gas and acid production. The bulbs became uniformly clouded and a slight deposit developed; the branches remained clear. No gas was produced. After 5 days incubation the bulbs were titrated, and the reaction was found to be neutral or slightly acid. The original reaction of the bouillon had been 0.5 to

TABLE I.

Resistance to Drying.

Strain No.	Temperature.	Time of exposure.	Growth appears in.	Motility in hanging drop.
	°F.		days	
192 (61st transfer)	70–75	1 hr.	3	+
192 (61st ")	70–75	2 hrs.	3	+
192 (61st ")	70–75	3 "		−
192 (61st ")	70–75	4 "		−
192 (61st ")	70–75	5 "		−
192 (61st ")	70–75	6 "		−
192 (61st ")	70–75	1 day.		−
149 (79th ")	70–75	1 hr.	4	+
149 (79th ")	70–75	2 hrs.		−

TABLE II.

Resistance to Heat.

Strain No.	Temperature.	Time of exposure.	Growth appears in.	Motility in hanging drop.
	°C.	min.	days	
192 (70th transfer)	56	5		−
192 (70th ")	56	10		−
192 (71st ")	55	5	3	+
192 (71st ")	55	10		−
149	55	5	3	+
149	55	10		−

0.6 per cent acid to phenolphthalein. The branch showed no acid production.

Vibrio fetus as it occurs in cultures has but little resistance to drying. Bits of linen thread impregnated with fresh active culture material resisted drying 2 hours at room temperature, but not 3 hours, as shown in Table I. Similarly resistance to heat is feeble. It is able to withstand a temperature of 55°C. for 5 minutes (Table II).

Agglutination Reactions.

The scarcity of positive differential characters of the spirilla isolated from fetuses leaves open the possibility that they may not all be alike and that many, if not all, are harmless invaders of the uterochorionic space and the fetal digestive and respiratory tracts. To test the first possibility agglutination with immune serum was resorted to at an early stage of the work. Rabbits were treated repeatedly with doses of living spirilla injected into the abdominal cavity. After 3 to 4 weeks blood was withdrawn and the serum used in the tests shown in Tables III to VI. Before applying the agglutination test it was necessary to await the time when the various strains could be made to multiply on agar slants without blood or bits of tissue so as to eliminate any possible interference on the part of animal tissues.

At this time one of us fortunately isolated a spirillum from the spleen of a young calf which, though culturally like the fetal strains, was nevertheless wholly different in its agglutination affinities. This culture which will be taken up in another publication was used in the tests as a control. It is designated as No. 174. Somewhat later another strain was isolated from the liver of a calf 8 weeks old and the spleen of a guinea pig inoculated with duodenal contents of the same calf. This agrees serologically with *Vibrio fetus* and appears in the tables as No. 321.

The serum used is from rabbits immunized against Strains 67, 174, 256, and 317. The antigens are saline suspensions of the several strains grown on sealed agar slants at 37°C. for 4 days. The salt solution used is 0.85 per cent. The volume of fluid in each final dilution is 2 cc. The culture control contains 1 cc. of the antigen suspension and 1 cc. of salt solution, the serum control 1 cc. of a 1:10 dilution of serum and 1 cc. of normal salt solution. After a period of from 2 to 3 hours at 37°C. readings are taken. The tubes are then placed in the refrigerator over night when the final readings are made.

Tables III to V show that the agglutination reactions are of the usual kind, except for very young strains which yield a more or less paradoxical reaction, in which the clumping is feeble or absent in

TABLE III.

A. Serum from a Rabbit Treated with Cultures of Vibrio 67.

Vibrio No.	No. of transfer.	Dilutions of serum.											Controls.	
		1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	1:20,480	Culture.	Normal serum.
67	64	C.*	C.	C.	C.	C.	C.	C.	+++[1]	+++[1]	++[1]	++	+	−	−
149	75	"	"	"	"	"	"	"	C.	+++[1]	+++	++[1]	++	−	−
159	72	"	"	"	"	"	"	"	"	C.	+++[1]	+++	++	Settling and clumps; cloudy.	−
174		−	−	−	−	−	−	−	−					−	−
179	71	C.	C.	C.	C.	C.	C.	C.	C.	C.	+++[1]	++[1]	++	−	−
192	?	"	"	"	"	"	"	+++[1]	+++	+++	++[1]	++	++	−	−
213	56			"	"	"	"	C.	C.	++[1]	++[1]			−	−
246	25			"	"	"	"	+++[1]	+++	++	+			−	−
251	23			+++[1]	"	"	"	+++[1]	+++	+++	++			−	−
256	21			C.	"	"	"	+++[1]	+++[1]	+++	+++			−	−
258	19			"	"	"	"	++	+	+	+			−	−
263	14			"	"	"	"	C.	C.	C.	+++			−	−
267	17			−	−	−	+++	+++[1]	"	++[1]	++			−	−
289	8		C.	+++	+++	+++	+++	++	++	++	−	−		−	−
290	8		"	C.	C.	C.	++	++	+	+	+	−		−	−

* In this and the following tables C. indicates complete agglutination, +++ nearly complete agglutination, ++ marked agglutination, + slight agglutination, ± doubtful, and − no agglutination.

[1] when following a symbol denotes a degree of agglutination between it and the next higher symbol.

TABLE III—*Concluded.*

B. Serum from a Rabbit Treated with Cultures of Vibrio 67.

Vibrio No.	No of transfer.	Dilutions of serum.								Controls.	
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	Culture.	Normal serum.
67	90	C.	C.	C.	C.	C.	C.	+++[1]	−	−	−
149	97	"	"	"	"	"	"	C.	C.	−	−
318	17	"	"	+++[1]	+++	+++	++	+	+	−	−
159	96	"	"	C.	C.	C.	C.	C.	C.	−	−
179	89	"	"	"	"	"	+++	++	+	−	−
289	25	"	"	"	"	+++[1]	+++	++[1]	++	−	−
317	18	"	"	"	"	C.	C.	C.	+++[1]	−	−
308	21	"	"	"	"	"	"	+++[1]	++	−	−
317	19	"	"	"	"	+++	++	+	±	−	−
267	39	"	"	"	"	C.	C.	+++[1]	+++	−	−
321	17	"	"	+++[1]	+++	++	+	−	−	−	−
321	13	"	"	C.	C.	+++[1]	+++	++	+	−	−
331	4	+++	+++	+++	++	+	−	−	−	−	−
333	3	++	++	++	++	++	++	−	−	−	−
339	3	+	++	++	++	++	+	+	+	−	−

TABLE IV.

Serum of a Rabbit Treated with Cultures of Vibrio 256.

Vibrio No.	Dilutions of serum.								Controls.	
	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	Culture.	Normal serum.
67	C.	C.	C.	C.	C.	+++[1]	+++	+++	–	–
149	"	"	"	"	"	C.	+++[1]	+++[1]	Slight deposit; cloudy.	–
159	"	"	"	"	"	"	+++[1]	+++[1]	" "	–
174	–	–	–	–	–	–	–	–	–	–
179	C.	C.	C.	C.	C.	+++[1]	+++	+++	–	–
192	"	"	"	"	"	C.	+++[1]	+++[1]	–	–
213	"	"	"	"	"	"	+++[1]	+++[1]	–	–
246	+++	"	"	"	"	"	C.	+++	–	–
251	–	–	+++	+++[1]	+++[1]	"	+++	+++	–	–
256	C.	C.	C.	C.	C.	"	C.	+++	–	–
258	"	"	"	"	"	"	+++[1]	+++[1]	–	–
263	–	+++	"	"	+++	++	+[1]	+	–	–
267	–	–	+	++	+++	C.	+++	++	–	–
289	+	++	+++	C.	C.	+++	++	–	–	–
290	–	–	+	+++	"	C.	+++	++	–	–
321a*	+++[1]	+++[1]	++	+	–	–	–	–	–	–
321b†	C.	C.	C.	+++	+++	+	–	–	–	–

* Liver strain.

† Guinea pig passage strain.

TABLE V.

Serum of a Rabbit Treated with Cultures of Vibrio 317.

Vibrio No.	No. of transfer.	Dilutions of serum.								Controls.	
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	Culture.	Normal serum.
317	20	C.	C.	C.	C.	C.	++[1]	+++[1]	+++	Slight sediment.	–
67	92	+++	+++[1]	+++[1]	++	±	–	–	–	–	–
174	95	–	–	–	–	–	–	–	–	–	–
213	71	C.	C.	C.	C.	C.	+++	++	+	–	–
267	42	"	"	"	+++	++	+	±	±	–	–
318	21	"	"	"	C.	+++	++	+	–	–	–
290	25	"	"	"	+++	++	+	±	–	–	–

TABLE VI.

Serum of a Rabbit Treated with Cultures of Vibrio 174.

Vibrio No.	Dilutions of serum.												Controls.	
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	1:20,480	Culture.	Normal serum.
67	—	—	—	—	—	—	—	—					—	—
149	—	—	—	—	—	—	—	—					—	—
159		—	Slight clearing.	Slight clearing.	—	—	—	—					Slight deposit; cloudy.	—
174	C.	C.	C.	C.	C.	C.	C.	C.	C.	C.	C.	+++[1]	—	—
179		—	—	—	—	—	—	—	—				—	—
192		—	—	—	—	—	—	—	—				Slight deposit.	—
213		—	—	—	—	—	—	—	—				—	—
246		—	—	—	—	—	—	—	—				—	—
251		—	—	—	—	—	—	—	—				—	—
256		—	—	—	—	—	—	—	—				—	—
258		—	—	—	—	—	—	—	—				—	—
263		—	—	—	—	—	—	—	—				—	—
267		—	—	—	—	—	—	—	—				—	—
289		—	—	—	—	—	—	—					—	—
290		—	—	—	—	—	—	—					—	—
308*		—	—	—	—	—	—	—	—				—	—
321a		—	—	—	—	—	—	—	—				—	—
321b		—	—	—	—	—	—	—	—				—	—
174		C.	C.	C.	C.	C.	C.	C.	C.				—	—

* This and the following tests were made at a later date.

the highest concentration and rises to a maximum to fall again. The older strains present the usual features of complete sedimentation with clarification of the supernatant fluid in the high concentrations, and gradual increase of clouding of the fluid in the lower ones. The tables indicate a close relationship between Strains 67, 256, and 317, if not identity, as the word is used in establishing a definite species of bacteria. Furthermore, the calf strain, No. 174, shows no agglutination affinities whatever with the fetal strains or with the other calf strain, No. 321.

SUMMARY.

Twenty-two fetal and two calf strains of spirilla have been studied chiefly with regard to the problem of identity. Twenty-one fetal strains are probably specifically the same. One fetal strain differs slightly from these, but in its agglutination affinities it belongs to the same group. Of two strains isolated from calves one has definite agglutination relations with the fetal strains, the other none. In the morphological and biological characters so far investigated all the strains agree closely with one another.

EXPLANATION OF PLATE 16.

Magnification, × 1,000.

FIGS. 1 to 3. *Vibrio fetus* (No. 159) stained to show the polar flagella.

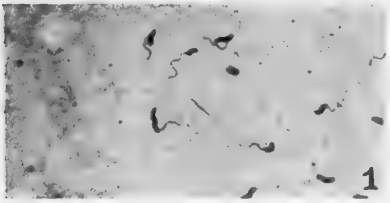
FIG. 4. *Vibrio fetus* (No. 213) stained in the same way.

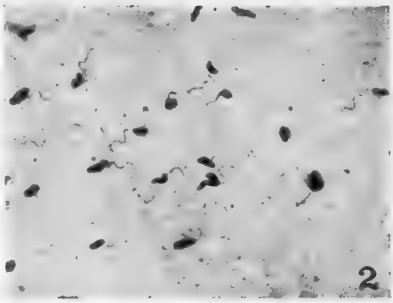
FIG. 5. Spirillum from a calf (No. 174) stained in the same way.

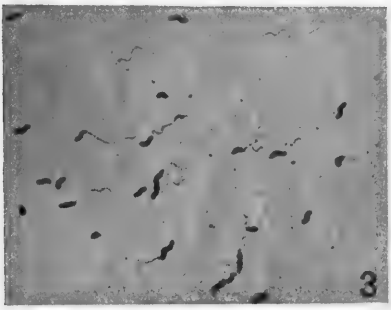
FIG. 6. Preparation from a culture of *Vibrio fetus* (No. 290), 10th transfer, showing long and short forms, some stained deeply and with granules, others more faintly. Dilute aniline water gentian violet.

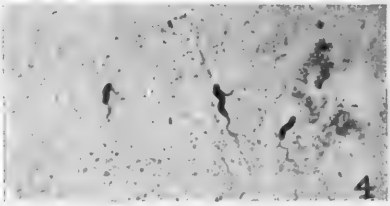
FIG. 7. Preparation from a culture of *Vibrio fetus* (No. 333) showing long forms. Stain as in Fig. 6.

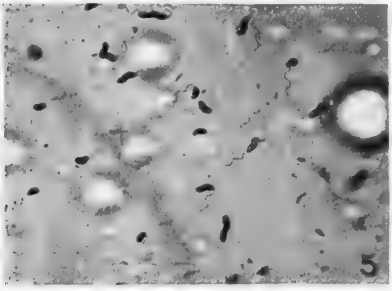
FIG. 8. Another field of the same preparation showing some capsular substance, unstained, sheathing the long forms.

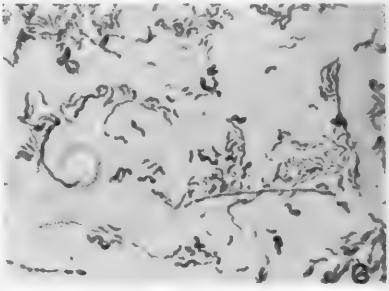


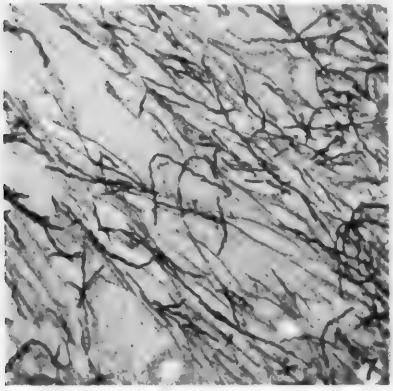


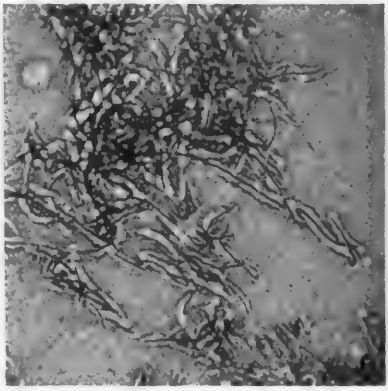












(Smith and Taylor: Morphology and biology of *Vibrio fetus*.)

THE ETIOLOGICAL RELATION OF SPIRILLA (VIBRIO FETUS) TO BOVINE ABORTION.*

By THEOBALD SMITH, M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Received for publication, June 3, 1919.)

Since the summing up of the data contained in a former communication on this subject[1] investigations have been continued upon material from the same herd. From August, 1918, to June, 1919, twelve additional cases of abortion in which spirilla were isolated from fetuses in pure cultures have been studied, making twenty-six in all. In one case a slightly divergent strain was present, whose significance cannot be defined. It appears, as a rule, in long filaments, although short forms are not lacking. Thus far, motility has not been detected. The case from which this form was isolated is included in Table I as No. 308. In another paper[2] data on the agglutination affinities of the various strains are given and it is there shown that this aberrant strain is serologically related to the regular type. The reader is also referred to an earlier paper[1] on culture methods employed. Emphasis needs to be put on the importance of cultures from the digestive tract and the lungs. If the fetus after expulsion breathes and swallows, both tracts are likely to be contaminated with miscellaneous bacteria and spirilla will be suppressed in the cultures. Even when the fetus is dead when expelled, if the mouth and nose should lie in water or other fluids these tracts may become contaminated. Cultures from spleen, liver, and kidneys of the fetus by themselves

* This and the three accompanying papers are interrelated inasmuch as the fundamental data are the same. Duplication of statements has been avoided as far as possible and the papers should therefore be considered together in estimating the accuracy of the conclusions formulated.

[1] Smith, T., *J. Exp. Med.*, 1918, xxviii, 701.

[2] Smith, T., and Taylor, M. S., *J. Exp. Med.*, 1919, xxx, 299.

TABLE I.

Fetuses from Which Spirilla Were Isolated. Guinea Pig Tests for the Presence of B. abortus.

Cow No.	No. of sire.	Length of fetus.	Date of abortion.	No. of pregnancy after purchase.	Cultures.							Guinea pig inoculations.			
					4th stomach.	Rectum.	Lung.	Spleen.	Liver.	Kidney.	Fetal fluids or swabs.	4th stomach.	Lung.	Meconium.	Placenta or uterine discharge.
		in.	*1918*												
289	8, 6, 2	20	Nov. 18	2	++* (Rumen ++.)	++	+	−−	−−	++		−	−	−	−
290	4, 9	24	" 19	3 (native).	+	+	++	++	++	++		−	−	−	−
308	?	31½	Dec. 30	1	−−	+++ (Small intestine −.)	++	−−	−−	−−		−	−	−	−−
			1919												
317	9	25	Jan. 27	2	+ and other forms.	++	++					−	−	−	
318	3, 7	26	" 27	2	++	+ and other forms.	+ and other forms.					−	−	−	−
331	9	8	Mar. 15	6 (native).	Two pure cultures of spirilla; one culture of paratyphoid; one culture of cocci and spirilla.							−			−−
333	2, 10	26	" 27	1	++	++ (Colon ++.)	++ and streptococci.	−−	− and bacilli.	−−		−	−	−	
339	1, 2	35½	Apr. 9	3	−− (Films +.)	−− (Films of small intestine +.)	++	−−	−− (*B. pyogenes* +.)	−−		−	−	−	−

347	2	25½	Apr. 23	4	++ (Rumen ++.)	++	++	+	−−	−−		−	−		−
351	7, 11	28	May 5	1	++ and *B. abortus*.	++	Miscellaneous bacteria.	+ and *B. abortus*.	Miscellaneous bacteria.	+ and *B. abortus*.		−	+	−	+
356	4, 8	26½	" 23	1	+++ (One tube contains a bacillus also.)	++ (Colon +.)	++		++	++		−	−	−	−
357	9	21¾	" 24	8 (native).	++ (Small intestine +.)	++ (Colon +.)	+ and contaminated.	Contaminated.	−	−	(Film +.)	−	−	−	−

* Each + stands for a pure culture of spirilla unless otherwise stated, each − for a negative culture or negative guinea pig test with reference to *B. abortus*.

cannot be depended on to bring out the presence of spirilla in the fetus.

The notes on the pathological changes in the fetuses are omitted, since they do not differ from those of the earlier cases reported.[1] No single abnormal condition or group of conditions in the fetus could be used to prognosticate the nature of the infecting organism.

In most cases the placenta was retained. In one (No. 289) it was reported to have been discharged but it was not preserved. A portion of the placenta of No. 318 was passed attached to the fetus. It represents about one-third of the entire mass. About one-half is markedly diseased. The cotyledons are of a dirty whitish color and covered with a thin deposit which is readily washed off by repeated baths of normal salt solution. The villi after washing stand up like normal villi but lack entirely the blood-red color. Towards the more normal cotyledons some are found which contain abnormal, whitish villi and tufts or clumps of the same among the normal blood-red ones.

The intercotyledonous chorion, where cotyledons are diseased, is usually thickened and roughened. The surface is mapped out into small 1 to 2 mm. slightly raised plaques. These feel like thickened epidermis. When scraped only very little comes away. The underlying tissue is more or less edematous in localized areas, usually around and under a diseased cotyledon. Films of the cheesy covering of cotyledons stained 10 minutes and 24 hours show numerous spiral forms with from $\frac{1}{2}$ to 4 or 5 complete turns. They are all of the same fineness as regards diameter, but the amplitude of curves and the form vary somewhat. This may be due to the drying.

Sections of fixed and hardened tissue show besides a general, quite marked edema of the chorion, localized masses of nuclear debris under the epithelium, now lost. This nuclear debris results from dense collections of leucocytes, probably polynuclear, which evidently form the hard plaques of the intercotyledonous areas of the chorion, mentioned above. There is, furthermore, a general diffuse infiltration of cells into the chorion. The cotyledons contain necrotic villi without cell infiltrations.

It was stated in the preceding article[1] that *Bacillus abortus* could not be detected in the fourteen cases from which *Vibrio fetus* was iso-

lated. The same is true of those tabulated here, with the exception of one case, No. 351. In cultures from this there were found for the first time both spirilla (vibrios) and *Bacillus abortus*. The former were present in cultures from contents of the fourth stomach, colon, rectum, spleen, and kidneys. *Bacillus abortus* appeared several days later in the fourth stomach, spleen, kidney, and rectum cultures. The tubes with contents of the colon contained only spiral organisms.

The absence of *Bacillus abortus* from such a large percentage of cases is of importance. The dropping of immature calves and fetuses of various ages can no longer be referred to *Bacillus abortus* without a bacteriological study of the fetus and placenta if obtainable, or else of uterine discharges obtained with swabs and examined microscopically and by inoculation of guinea pigs. Outbreaks of abortion due solely to *Bacillus abortus* very probably occur when a herd is first attacked and therefore highly susceptible. After a time the acquired immunity modifies the conditions materially and *Bacillus abortus* becomes a relatively unimportant factor in all but first pregnancies.

Vibrio fetus may be either a true agent causing disease of the fetal membranes, or it may be simply an invader from the more external genital tract, or from the blood after the fetus has been injured or killed by other non-bacterial agencies. The simplest method to prove the truth of one or the other hypothesis is to inoculate pregnant cows. This procedure is not so easily carried out, however. In addition to the difficulties encountered in obtaining non-immune and non-infected pregnant cows is the probable attenuation of the vibrio under cultivation. This, as is described elsewhere, is subject to changes under cultivation, such as a tendency to form mucoid masses, and an adaptation to culture media without blood or bits of tissue. The quantity of growth tends to increase with successive transfers. Changes in growth characters as extensive as these most probably involve other changes, among which virulence stands first. Unfortunately, laboratory animals have so far proved refractory and cannot serve as tests of virulence. If very early transfers are inoculated into cows, the danger of introducing some hypothetical ultramicroscopic virus is much greater than in later transfers. Filtration

does not remove this possibility, since the minute spiral organisms, especially the short forms, might pass through the pores of the filter. With these difficulties in view the writer has been able to test *Vibrio fetus* upon four pregnant cows. The records of these cases are as follows:

Cow 295.—Received Nov. 21, 1918. Black Holstein of unknown age, supposed to be pregnant, according to physical examination. Reacts to tuberculin.

Dec. 10. Cow received into the left jugular vein the following suspension. Four agar slants, sealed, of vibrio (Cow 267, 17th transfer, inoculated Dec. 6) were used. The condensation water was first removed with pipette, then the agar moved about after adding some fresh bouillon to dislodge organisms growing between agar and glass. In all, about 10 cc. of a faintly clouded fluid were obtained. A hanging drop of this fluid shows rather long spirilla with feeble motility. Before the date of the injection the maximum range of temperature during the day was from 38.4°C. at 8 a.m. to 39.4°C. at 5.15 p.m. On the day of injection, which was made at noon, the late afternoon temperature was 40.6°C. On the following day it had fallen to normal.

On the assumption that the culture injected might have lost some of its virulence a second injection of a more recently isolated strain was made Jan. 8, 1919. Growth from sealed agar slants (Fetus 289, 7th transfer, 6 days old) was suspended in plain bouillon. About 9 cc. of a feebly clouded fluid were injected into a jugular vein. There was hurried respiration following the injection and some coughing. The maximum afternoon temperature was 39.8°C. Between the date of injection and the birth of a calf on Feb. 6, the morning temperature was either slightly higher than the evening temperature or else the same. The maximum morning elevation was 39.5°C., the usual temperature 39–39.3°C.

Feb. 6. After normal labor cow gave birth to a living calf. The placenta was still retained after 24 hours and the major portion of it was removed manually.[3] A thick, turbid, reddish discharge with small coherent mucoid masses in it was caught in a sterile bottle by the attendant several hours after birth of calf. Fresh and stained films showed presence of cells resembling polynuclear leucocytes but with chromatin chiefly on margin and central portion nearly unstained and in most cases containing a small number of organisms, some of which were distinctly spiral, others of same diameter, but spiral form not demonstrable in them. The groups of leucocytes were in part held together by amorphous masses staining blue. Many isolated spirillar forms of one or two wave lengths present. Other bacteria scarce.

Of the placenta removed Feb. 7, the intercotyledonous areas of chorion have the minute network of vessels conspicuously injected. Small masses of a yellowish exudate, of pin-head size, sprinkled over the surface. These masses con-

[3] The placenta was removed by Dr. Ernest W. Smillie and Dr. Ralph B. Little.

sist of amorphous material staining blue, remnants of cells and miscellaneous bacteria. The subchorionic tissue is not edematous. Besides these abnormal conditions, the chorion presented several areas which were thickened, opaque, whitish and slightly nodular, firm and leathery. The cotyledons vary much in appearance. None has the blood-red color of the normal mature placenta. In all there are yellowish areas, occupying more or less of the cotyledon and supplanting the normal color. This yellowish color is due largely to deposits or exudates like those described above. When a cotyledon is rinsed repeatedly in normal salt solution, the wash water becomes heavily clouded and filled with flocculi. After three or four changes these are removed and there remain in the cotyledon a variable number of yellowish villi among the dark or light red ones, which are the result of necrosis. These may form an outer zone only or occupy most of the cotyledon. In none of the many films examined were bacteria resembling *B. abortus* found. Up to the time this cow was removed for slaughter, Feb. 12, a slender mass of placental tissue about 2 feet long remained hanging from the vulva. Unfortunately, through some mistake at the abattoir, the uterus was not reserved.

In sections from the placenta there is a complete loss of chorionic epithelium. There is extensive cell infiltration with subsequent necrosis and nuclear disintegration in foci along the subepithelial layer of the chorion and in the villi. These and their branches are densely filled with nuclear debris. Occasionally a vessel is recognizable, also densely filled. The appearances indicate an active polymorphonuclear infiltration followed by necrosis. Microorganisms not definitely seen.

The calf of No. 295 appeared somewhat under weight at birth. On the 4th day it weighed 75 pounds. At birth the feet were somewhat soft and hoofs not fully developed. In all other respects the calf was normal and remained free from scours during the 6 days it was under observation.

To determine whether this cow might harbor *B. abortus* as a cause of the disease of the placenta, two guinea pigs were inoculated with uterine exudate obtained several hours after birth of calf. Both guinea pigs were normal when killed 48 days after inoculation and cultures were negative Before the first inoculation on Nov. 28, samples of blood were withdrawn from jugular vein and the serum was tested for specific agglutinins towards *B. abortus*. Only in dilution of 1: 10 was clumping observed. On Jan. 27, a second sample was withdrawn. This also was negative. Tested against *Vibrio fetus*, a very good agglutination was obtained with two older strains but not with recent strains, a condition quite regularly observed (Table II).

On Dec. 2, 1918, about 100 cc. of milk were obtained from the udder. To this were added 50 cc. of salt solution and the mixture was centrifuged at high speed for 20 minutes. Three guinea pigs were inoculated by injecting 7 cc. of the deposit and lowest strata of fluid into the peritoneal cavity of each. After 57 days the animals were killed with chloroform. There were no lesions suggestive of the presence of *B. abortus* and the cultures from the spleens remained free from growth.

The evidence is thus quite conclusive that *Bacillus abortus* was not responsible for the lesions. The microscopic examination of the early postpartum discharges showed many spirilla both free and within leucocytes. The placenta was frankly diseased and the character of the lesion, infiltration and exudation of leucocytes, corresponded with the lesions found in earlier cases, associated with spirilla. The following case is not so convincing as to the rôle of spirilla in producing disease of the chorion but the evidence is against an infection with *Bacillus abortus*.

TABLE II.

Cow 295. Agglutination Test.

Strain from Cow No.	No. of generation or transfer.	Dilutions of serum.*							
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560
67	78	C.†	C.	C.	C.	C.	C.	+++	++
179	81	+++	"	"	"	"	+	−	−
267	25	−	−	+	−	−	−	−	−
289	11	−	−	−	−	−	−	−	−

* Serum from blood withdrawn Jan. 27, 1919. The tubes were kept at 37°C. for 3 hours, then in the refrigerator at about 6°C. over night.

† In this and the following table C. indicates complete clumping, and clearing of the fluid, − no change. The plus signs represent grades of clumping.

Cow 296.—White Holstein, obtained with No. 295. Assumed to be pregnant according to physical examination. Reacts to tuberculin.

Beginning Nov. 25, 1918, the temperature was taken twice daily. The highest morning temperature up to Dec. 10, the day when the animal was inoculated, was 39.2°C., the highest evening temperature 39.6°C. On Dec. 10, the growth in four sealed agar slants of spirilla from Cow 289, 4th transfer, 4 days old, was suspended in bouillon. About 10 cc. of a faintly clouded suspension were injected into the left jugular vein at noon. On this day the late afternoon temperature was 41.2°C. Next day the temperature was normal. Attendant reported violent jerky movements in abdomen of cow.

A normal calf was born early Dec. 19, 9 days after inoculation. At 9 a.m. the placenta was not completely discharged. It extended from vulva to floor, (the animal standing) where most of it lay in the bedding. A portion, including half a dozen cotyledons, which had not touched the floor was removed for examination. The intercotyledonous areas of chorion have all vessels conspicuously injected. The larger ones are cyanotic in appearance. Resting on these areas are soft, orange-colored masses of pin-head size which crush like soft cheese

between cover-slips. They consist of cell debris, fat globules, and probably leucocytes. When stained, these masses are seen to consist of polynuclear leucocytes, some epithelium, and cell debris, bound together with a cement-like substance, staining blue in alkaline methylene blue. A few spirilla-like forms detected in films of these masses after considerable search. The cotyledons in general are free from necrotic villi and the latter appear normal when covered with water. Over a number of cotyledons, however, there is the same flaky exudation, even more abundant than over the intercotyledonous areas. Films of this exudate show a few curved forms besides the cellular and amorphous masses already described. Sections of fixed and hardened portions of placenta indicate in general a still normal tissue. In slides from four different regions, however, there are a few small foci of dense polynuclear cell infiltration. One of these surrounds a thrombosed vessel in a villus.

Cultures from the exposed placenta became overgrown with a variety of bacteria. Spirilla not detected in the mixed growth. On Dec. 25, pasty, whitish, semitranslucent masses were found in bedding. These probably came from the uterus. Films showed pus cells and immense numbers of minute cocci and rod-like forms.

In order to determine whether *B. abortus* was associated with the placental lesions, three guinea pigs were inoculated with scrapings of the placenta, ground in sand and suspended in salt solution. After 43 days they were killed. There were no lesions suggesting *B. abortus* and the spleen cultures remained clear.

To determine still further the presence or absence of *B. abortus* a sample of blood was collected Nov. 28, and the serum tested against *B. abortus*. Only in a dilution of 1:10 was clumping recognizable. A second sample withdrawn Jan. 13, 1919, gave a similar result. The titer of the same serum was tested against four strains of spirilla (Table III).

On Dec. 2, 1918, about 50 cc. of milk were obtained from the udder, centrifuged, and of the lowest layer about 15 cc. were injected into the peritoneal cavity of three guinea pigs, 5 cc. into each animal. One guinea pig died in 39 days. There was extensive plastic peritonitis causing adhesion to one another of all the abdominal organs. The two remaining animals were killed with chloroform in 57 days and were found normal. Cultures from spleen remained free from growth.

The two following cases, both inoculated with strains of spirilla, were entirely negative.

Cow 334.—Received Mar. 20, 1919. Jersey grade, about 7 years old. Pregnant according to physical examination. Reacts to tuberculin.

Mar. 31, noon. Cow received into left jugular vein a suspension of spirilla isolated from Fetus 331. The cultures used were of the 3rd generation (or transfer) in sealed tubes of slanted agar containing bits of guinea pig spleen and some calf serum water. Growth after 4 days incubation feeble, but charac-

teristic. It was washed off with salt solution, bouillon added to 4 cc., and gently centrifuged to throw down minute clumps of agar, etc. The resulting suspension contained active spirilla. The temperature taken twice daily from the time of arrival fluctuated between 38° and 39°C. except on the afternoon of the day of injection, when the late afternoon temperature was 40.6°C.

An agglutination test against *B. abortus* was feebly positive at 1:20. The cow was kept under observation until Apr. 28, when she was slaughtered. The fetal membranes were normal in appearance. The fetus was 31 inches long. The only departure from the normal was the presence of a small quantity of reddish, clear fluid in the thoracic and abdominal cavities and a slight interlobular edema of the lungs. Cultures from fetal fluids (amniotic, allantoic, scrapings from chorion) and from fourth stomach of fetus were sterile after 2 weeks of incubation.

TABLE III.

Cow 296. Agglutination Test.

Strain from Cow No.	No. of generation or transfer.	Dilutions of serum.*								Controls.	
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	Culture.	Serum.
67	79	C.	C.	C.	C.	+++	−	−	−	−	−
179	81	"	"	"	"	+++	−	−	−	−	−
267	24	−	−	−	−		−	−	−	−	−
289	10	+	+	+	−	−	−	−	−	−	−

* Serum from blood drawn Jan. 13, 1919. Tubes kept at 37°C. for 3 hours, then in refrigerator at about 6°C. over night. The control culture was suspended in salt solution. The serum control contained 5 per cent serum.

Cow 335.—Received with No. 334. Jersey grade, 9 to 10 years old. Pregnant. Reacts to tuberculin.

Mar. 31, noon. Inoculated with spirilla from Fetus 317, 13th transfer, on slanted agar. The cultures were 4 days old. Growth fair. The spirilla were removed in the same manner as for Cow 334. Active spirilla present in the final suspension. The injection was made into the left jugular vein. Within a few minutes the cow began to breathe rapidly and cough. This lasted about 2 hours. The temperature, which had been fluctuating between 38.4°C. in the morning and 39.4°C. in the late afternoon, rose to 40.6°C. on the day of injection and was normal again next day. On the same day a slight discharge from vagina was noted. A guinea pig inoculated with it showed no disease, 6 weeks later.

Agglutination tests made with *B. abortus* on Mar. 27 showed a positive clumping up to dilutions of 1:160. The cow was slaughtered with No. 334, Apr. 28, and the uterus with contents removed to the laboratory. There was no evidence of disease of the chorion. Cultures from the several fluids (allantoic, amniotic) and scrapings from chorion were negative. The fetus was 26¾ inches long. Its

organs were normal with the exception of marked edema of the walls of gall bladder and the mesentery of duodenum carrying the common bile duct. Cultures of fourth stomach fluids remain sterile.

A sample of milk obtained Mar. 27 and tested on guinea pigs was later shown to be infected with *B. abortus*. This explains the agglutination titer of 1:160 towards *B. abortus* and makes it certain that this cow had passed through the abortion disease in some earlier pregnancy. It also shows that the udder may be infected with *B. abortus* and the fetal membranes and fetus remain normal. The agglutination reactions of the blood of Cows 334 and 335, taken Apr. 24, towards their own and one other strain of spirilla were quite irregular and the results are therefore withheld for further study of this test.

The foregoing experimental tests of the pathogenic rôle of *Vibrio fetus* cannot be regarded as final, but as far as they have been carried they give strong evidence that it is an etiological factor in bovine abortion. Spiral forms from four different cases were inoculated (Nos. 267, 289, 317, and 331). Only the two cases inoculated with those from Case 289 may be considered positive. Whether this negative outcome in the two remaining cases is due to decline or absence of virulence or to varying resistance on the part of the cows must be determined by additional experiments of the same kind.

SUMMARY.

The isolation in pure culture of a definite morphological entity (vibrio or spirillum) with practically the same biological characters from a series of cases of the same clinical complex (abortion) establishes a presumption in favor of the specific identity of the organisms and also in favor of the inference that such organisms are etiologically related to the diseased condition. This presumption is strengthened by the fact that disease of the fetal membranes followed the injection of pure cultures of this organism in two out of four cases.

THE BACTERIOLOGY OF BOVINE ABORTION, WITH SPECIAL REFERENCE TO ACQUIRED IMMUNITY.

By THEOBALD SMITH, M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Received for publication, June 3, 1919.)

Since the general confirmation of Bang's discovery of a characteristic bacillus, *Bacillus abortus*, associated with abortion in cattle in different countries and the successful production of disease of the fetal membranes through infection of pregnant cows with *Bacillus abortus* in pure culture, it has been taken for granted that Bang's bacillus was the sole infectious agent, and further research into the etiology has given way to the study of methods of diagnosis, of vaccines, and their application.

The bacteriological study of cases of abortion as they occurred in a large herd in which a fair proportion of the stock was bred on the spot with accessions from other herds from time to time, continued over a period of $2\frac{1}{2}$ years, has shown that while *Bacillus abortus* may be the sole agency of abortion in certain herds, this is clearly not true for the herd under investigation.

In view of this somewhat unexpected result it seemed desirable to go over the data pertaining to the various cases in some detail, first, to see how far the bacteriological results could be depended upon to give information concerning the nature of the infectious process leading to death and expulsion of the fetus, and second, to note the bearing of the bacteriological data on the acquisition of immunity by the cow against later infection.

Methods.

The methods used for determining the presence or absence of infectious agents in the fetus and fetal membranes were those directed towards finding *Bacillus abortus* and spirilla (*Vibrio fetus*). Bits of

tissue from lungs, spleen, liver, and kidneys, and several drops of fluids from the different sections of the digestive tract of the fetus were added to slanted agar and the tubes sealed with sealing-wax. At the same time guinea pigs were inoculated with salt solution suspensions of contents of the digestive tract, portions of lungs, fetal membranes, and of swabs from the uterus whenever obtainable. For the details of the method of using guinea pigs and the results obtainable, the reader is referred to the communication by Smillie.[1]

In general, cultures will not succeed unless the amounts of fluid or tissue transferred to culture tubes are adequate. Usually bits of tissue the size of small beans or fluids of equivalent amount are sufficient. But even with these the growth may be limited to the condensation water and the adjacent portion of the slant on account of the few bacteria contained in the material.

As to the relative delicacy of the two methods—cultures direct from the fetus and guinea pig inoculations—in revealing the presence of a very few abortion bacilli, it may be stated that in most cases direct cultures and guinea pig inoculations agreed in being either both positive or both negative. In a few cases, however, in which the inoculation of guinea pigs failed to produce either manifest lesions or positive cultures from the spleen, the direct cultures showed the presence of a very few abortion bacilli. The method of direct culture may therefore be considered superior to the guinea pig test under conditions assuring pure cultures.

Under other conditions the use of guinea pigs must replace largely the cultures. Fetuses over 7½ months old may be born alive. In that case they inspire a little air and swallow some saliva or bedding before they die. The invasion of miscellaneous bacteria goes on very rapidly and cultures with lung tissue or fluids from the stomachs will usually be overgrown with a variety of forms. Guinea pigs inoculated with such material may show the presence of *Bacillus abortus* unless the invasion has gone too far, when the guinea pigs may rapidly succumb to a miscellaneous infection in which pathogenic anaerobes figure largely.

To obtain pure cultures of *Bacillus abortus* from the fetal mem-

[1] Smillie, E. W., *J. Exp. Med.*, 1918, xxviii, 585.

branes, the indirect method of inoculating guinea pigs is usually the only one successful, unless the placenta is promptly discharged and, if soiled, the diseased portions are repeatedly washed in sterile water or salt solution. This procedure removes much of the infection itself in the shape of infected epithelium and phagocytic cells, but it leaves enough to make isolation of *Bacillus abortus* possible. The material is transferred to an agar slant and well distributed over the surface. A loopful from this is transferred to a second, and so on to a third or fourth and the tubes are sealed.

When the placenta remains within the uterus 24 hours or longer, the miscellaneous infection becomes so great that guinea pigs inoculated with uterine fluid or suspensions of placental tissue rarely survive a septic infection resembling that due to malignant edema or *Bacillus welchii*, unless very small doses are injected and three or four guinea pigs used. Retention of the placenta *in utero* following abortion is the rule, and therefore cultures and guinea pig inoculations of discharges are likely to fail because of the predominance of other bacteria. Even when the placenta is discharged in part it may be in a condition which precludes the successful isolation of *Bacillus abortus*.

The question then arises how far the results obtained from cultures and guinea pig tests of fetal tissues and fluids can be depended on to assure a diagnosis. In other words, is the fetus invariably infected with *Bacillus abortus* when the placenta is involved? If not, what is the percentage of abortions in which *Bacillus abortus* fails to be demonstrable in the fetal organs by the method described?

This, like other questions involving bacteriological methods, can be answered only through cumulative data. It is impossible to subject the entire fetus to bacteriological tests, and the bacteria may be so scarce or so unevenly distributed in the digestive and respiratory tracts as to escape the culture tube. The data on which this paper is based contain a small percentage of cases in which both fetus and placenta were cultured and tested on guinea pigs. This material will be fully discussed in another publication, and we can merely give results here which go to show that in perhaps not more than one out of twenty-five cases were cultures from the fetus negative and guinea pig inoculations from placental tissue or uterine discharges positive.

It may on the whole be accepted, therefore, that only a very small proportion of abortions due to *Bacillus abortus* occur before the fetus has been invaded. Even in the case of certain fetuses born alive which survived from 1 to 7 days, *Bacillus abortus* was isolated from the lungs through guinea pigs. Owing to the presence of septic organisms the intestinal contents in such cases cannot be inoculated into guinea pigs without inducing a rapidly fatal septic infection in most animals.

Grouping of Cases According to the Associated Bacteria.

In the tables the cases, for convenience, have been divided into four groups: (1) the cows raised within the herd, from which *Bacillus abortus* was isolated; (2) the cows introduced into the herd from without, from which *Bacillus abortus* was isolated; (3) cases of infection with *Vibrio fetus*; (4) cases of abortion in which cultures remained sterile and guinea pig inoculations negative, or which were associated with other agencies such as *Bacillus pyogenes*.

In Table I there are 37 cases from which *Bacillus abortus* was isolated. Of these, 26 were first pregnancies, 7 were second pregnancies, 3 were third pregnancies, and 1 was a fourth pregnancy. Of the cows aborting in the second pregnancy, 4 aborted both times and 1 had a normal first gestation. Of the 3 cows aborting in the third pregnancy, one is recorded as having aborted in the second pregnancy. The other 2 have no record of previous abortions. One aborting in the fourth pregnancy has no record of earlier abortions.

In Table II are brought together all purchased cows whose fetuses or calves or fetal membranes were found infected with *Bacillus abortus*. Some of these cows had given birth just before purchase. Some gave birth soon after purchase. In the third column are given all calvings between the time of introduction into the herd and the abortion or premature delivery of calves. If the period between acquisition and the first calving thereafter is a month or less, the next pregnancy may be considered the first in which infection with *Bacillus abortus* became possible in the new surroundings. In such cases, therefore, the second pregnancy has been designated the first in the table.

TABLE I.

Native Cows, Aborting or Giving Birth to Living Calves from Which B. abortus Was Isolated.

Case No.	Length of fetus.	No. of calving.	Remarks.
2	46 cm.	2	Aborted both times.
16	Destroyed.	1	Placenta inoculated.
17	27 in.	1	
19	Calf alive.	2	
21	25 in.	1	
22	23 "	1	
26	?	1	
30	Nearly mature.	3	2 wks. premature. No record of earlier abortions.
31	27 in.	1	
33	26 "	1	
41	Nearly mature.	1	
78	28 in.	1	
86	21½ "	1	
89	Alive.	1	
105	19½ in.	3	Aborted also in second pregnancy.
120	14 "	2	First calf normal.
134	Calf alive.	1	Placenta inoculated.
146	Fetus not found.	1	" "
150	30½ in.	1	*In utero.*
153	2 cm.	2	
164	Alive.	1	
180	33 in.	1	Fetus lived several hrs.
200	21 "	1	
203	Alive.	1	
206	28 in.	1	
210	Calf alive.	1	
215	" "	1	
224	" "	1	
227	" "	1	
229	(80 lbs.)	1	
266	31 in.	3	No record of earlier abortions.
285	28½ "	1	
298	34 "	1	
301	Alive.	2	Calf killed when 7 days old. Cow aborted in Mar, 1917. (See No. 21.)
313	29 in.	4	No record of former abortions.
316	22¼ "	2	Aborted both times.
342	25¼ "	2	" " "

This group illustrates the fact, already recognized by owners of herds, that cows introduced into an infected herd from a clean herd will abort sooner or later and in this respect act as heifers raised in the herd. They have no protecting immunity. Among the twenty-five cases of Table II are sixteen which may be considered as aborting at the first opportunity; *i.e.*, during what may be called their first pregnancy in the new herd. Seven aborted in the second pregnancy and only two in the third.

Taking together the two groups of 62 cases from which *Bacillus abortus* was isolated either directly or through guinea pigs or with both methods, we find the following classes according to the number of pregnancies: Abortion at first pregnancy, 42; of these 26 were native heifers and 16 purchased cows. Abortion at second pregnancy, 14; of these 7 were native and 7 purchased cows. Abortion, at third pregnancy, 5; 3 were native cows and 2 purchased.

The group of 26 cases from which pure cultures of spirilla have been obtained has been tabulated in other publications.[3,3] Of these only 3 were native cows. The fetuses of these represented the third, sixth, and eighth pregnancy respectively. Among the purchased cows 6 cases were first, 9 second, 5 third, and 3 fourth pregnancies since purchase.

The fourth group of cases from which neither *Bacillus abortus* nor *Vibrio fetus* was isolated forms the most miscellaneous group and one most difficult to analyze. Of the twenty-one cases sufficiently studied through cultures, guinea pig inoculations, and the histological material to deserve a place in the table, one or two may possibly belong to the group of *Bacillus abortus*. Several may come within the group of *Vibrio fetus;* two were cases of infection with *Bacillus pyogenes* in which the preceding pregnancy had been cut short by *Bacillus abortus* infection. In eight, miscellaneous, rapidly growing bacteria appeared in the cultures. Of these several may be regarded as cases in which bacteria gained entrance immediately after expulsion, the fetus being large enough to have lived a short time after birth. One was clearly a case of asphyxiation at birth (No. 126) and one regarded by the

[2] Smith, T., *J. Exp. Med.*, 1918, xxviii, 701.
[3] Smith, T., *J. Exp. Med.*, 1919, xxx, 313.

TABLE II.

Purchased Cows, Aborting or Giving Birth to Living Calves from Which B. abortus Was Isolated.

Case No.	Date of purchase.	Date of calvings after purchase.	Date of abortion.	Size of fetus.	No. of calvings after purchase associated with *B. abortus*.
			1917		
20	Nov. 25, 1915		Mar. 16	Alive.	1
91	Apr. 19, 1917		June 5	30 in.	1
111	Nov. 20, 1915	Sept. 29, 1916	July 16	38 "	2
129		Oct. 2, 1915	Aug. 22	Alive.	2
		" 28, 1916			
131	July 14, 1914	July 9, 1915	" 30	28 in.	3
		Aug. 12, 1916			
			1918		
205	Dec. 14, 1916		Feb. 19	Twins; 35 and 36½ in.	1
214	Apr. 19, 1917	Apr. 24, 1917	Mar. 15	Alive.	1
222	July 25, 1916		Apr. 11	"	1
226	Apr. 3, 1916	Apr. 22, 1917	" 12	"	2
253	Aug. 17, 1917	Oct. 1, 1917	June 13	25 in.	2
270	Oct. 12, 1916	" 16, 1916	Aug. 22	29½ "	2
		Sept. 6, 1917			
273	Nov. 13, 1917	Nov. 20, 1917	" 27	16 "	1
275	Apr. 19, 1917	May 26, 1917	Sept. 2	16¼ "	1
276	Nov. 20, 1915	1917	" 4	20½ "	2
278	" 10, 1917		" 19	14½ "	1
281	" 13, 1917		Oct. 4	30½ "	1
305	Aug. 17, 1917	Sept. 16, 1917	Dec. 24	21½ "	1
			1919		
309	Apr. 6, 1918		Jan. 2	(58 lbs.)	1
311	Aug. 19, 1917		" 7	(38 ")	1
325	Nov. 13, 1917		Feb. 12	36 in.	1
328	Apr. 20, 1917	Aborted Apr. 13, 1918	Mar. 9	31 "	2
329	" 18, 1918		" 11	30 "	1
343	Oct. 12, 1918		Apr. 13	14½ "	1
350	Feb. 9, 1917	Aborted in Nov., 1917, and in May, 1918.	" 30	Alive.	3
351	May 12, 1918	May 25, 1918	May 5	28 in.	1

TABLE III.

Cases of Abortion from Which neither B. abortus nor Vibrio fetus Was Isolated.

Case No.	Length of fetus.	No. of pregnancy.	Purchased or raised.	Date of abortion.	Cultures from.							Remarks.
					Stomach.	Small and large intestines.	Meconium.	Lungs.	Liver.	Spleen.	Kidneys.	Fetal membranes.	
				1916									
4	(8 mos.)	?	Raised.	Apr. 1		—*		—	—		—		No earlier abortions.
35	16½ "	1	Purchased.	" 14				—	—		—		
				1917									
103	4 in.	3	Raised.	June 24								2 g. p.—	No earlier abortions.
122	24 "	1	Purchased.	July 31	Moulds. g. p.—	Moulds. g. p.—			Cocci.	—	—	g. p.—	
126	(75 lbs.)	2	" Sept. 4, 1915.	Aug. 15	— g. p.—	— g. p.—		—	—	—	—	2 g. p.—	Calf probably asphyxiated during birth.
143	(35 ")	1	Purchased Aug., 1917.	Oct. 11	g. p.—			—	—	—	—	2 g. p.—	Cow said to have been fighting few days before abortion.
158	12½ in.	1	Purchased Feb. 16, 1917.	Nov. 6								3 g. p.—	Calved Mar. 10, 1917. Probably a case of spirillum infection.
				1918									
201	34 "	2	Purchased 1917.	Feb. 12	Cultures overgrown.		— 2 g. p.—	Cultures overgrown. 2 g. p.—	—	—	—		Lungs partly inflated.
202	15½ "	4	Purchased Sept. 28, 1917.	" 13	—		— g.p.—	— g. p.—	—	—	—		No record of earlier abortion.

211	23 in.	4	Raised.	Mar. 13	— g. p.—		— g. p.—	—	—	—	—		Preceding calving 2–3 wks. premature (No. 30). *B. abortus* isolated at that time.
259	20 "	2	"	July 18			g. p.—	*B. pyogenes.* g. p.—	*B. pyogenes.*		*B. pyogenes.*		Aborted Oct. 22, 1917 (No. 146).
261	30½ "	6	Purchased Sept. 28, 1912.	" 26	— g. p.—		— g. p.—	— g. p.—	—	—			No record of earlier abortions. Lungs not inflated.
262	29 "	1	Purchased Dec. 5, 1916.	" 28	Streptococcus. g. p.—		— g. p.—	Colon bacillus. g. p.—	—	—	Heavy growth.		Lungs not inflated.
265	20 "	4	Raised.	Aug. 4			— g. p.—	Cultures contain *B. coli* types. 2 g. p.—					Calved Feb. 27, 1916. Aborted Aug. 30, 1916. Calved Dec. 20, 1917.
277	32 "	2	"	Sept. 11	Cultures overgrown.	Cultures overgrown.	— g. p.—	Moulds. g. p.—	—	—	—		Premature calf, 7½ mos. Alive. Cow calved normally Aug. 30, 1917.
279	8 "	2	"	" 27				— g. p.—				g. p.—	Aborted Jan. 20, 1918 (first pregnancy). Not studied.
282	Alive.	5	Purchased Aug. 21, 1912.	Oct. 8	Cultures overgrown. g. p.—		— g. p.—	Cultures overgrown. g. p.—					2 wks. premature.

* — indicates negative cultures; g. p. —, negative results with reference to *B. abortus* following the inoculation of guinea pigs.

TABLE III—*Concluded.*

Case No.	Length of fetus.	No. of pregnancy.	Purchased or raised.	Date of abortion.	Cultures from. Stomach.	Small and large intestines.	Meconium.	Lungs.	Liver.	Spleen.	Kidneys.	Fetal membranes.	Remarks.
				1918									
291	32 in.	4	Raised.	Nov. 19	*B. pyogenes.* g. p.—		— g. p.—	*B. pyogenes.* g. p.—	*B. pyogenes.*			g. p.—	No record of previous abortions. Fetus dead some time before expulsion.
300	35 "	6	Purchased Oct. 10, 1911.	Dec. 7	— g. p.—	Stomach—	— g. p.—	— g. p.—	—	—	Cultures overgrown.		Lungs not inflated.
				1919									
327	36 "	1	Purchased Aug. 10, 1917.	Feb. 18	Cultures overgrown. g. p.—		— g. p.—	— One culture overgrown. g. p.—	—	—	—	g. p.—	
344	36½ "	3	Purchased Aug. 20, 1916.	Apr. 12	— g. p.—	Stomach—	—	— g. p.—	—	—	Contaminated.	g. p.—	Calved in 1916 and 1917.

attendants as traceable to fighting several days before expulsion of fetus (No. 143). Seven may be considered bacteria-free or sterile. How many of these are due to injury, to toxic substances, and to food factors cannot be stated. The importance of these cases, because not associated with *Bacillus abortus*, has warranted a rather detailed publication of the data in Table III.

In this group too there are no first pregnancies. Those marked first in the table refer to purchased cows and are at least second pregnancies. Thirteen are purchased and eight native cows. The pregnancies during which the fetuses were dropped prematurely range from first to sixth.

Summing up the bacteriological data from another point of view we have 47 cases from which *Bacillus abortus* was not isolated. Among the latter were 25 cases associated with *Vibrio fetus* and 1 case containing a related but not identical spirillum; 2 with *Bacillus pyogenes*; 7 with sterile digestive and respiratory tracts; and 7 with only cultures of the digestive tract and lungs fertile, due chiefly to aspiration and swallowing of miscellaneous bacteria at birth.

Acquired Immunity to Bacillus abortus.

A statement frequently made in veterinary literature and by stock owners is that cows gradually lose the tendency to abort. This tendency is in harmony with the nature of the abortion disease,—an invasion of the fetal membranes, more particularly the chorion, by infectious agents, followed by multiplication of the latter and more or less injury to the tissues attacked.

The rapidity with which immunity is acquired varies considerably. Holth[4] quotes one observer as giving the experience of thirty veterinarians to the effect that cows tend to abort two or three times in succession. He quotes another observer as claiming that 68 per cent of aborting cows aborted but once, and Bang as stating that of 83 aborting heifers only 20 aborted next time. In most infectious diseases the young are attacked in largest numbers. Hence we would expect the largest number of abortions among primiparæ (heifers). But even here observations differ. Holth quotes an observer as

[4] Holth, H., *Z. Infektionskrankh. Haustiere*, 1911, x, 342.

stating that only 36 per cent of the cows under his care calved normally, whereas 45 per cent of the heifers did so.

In the tables it is evident that abortion is essentially a disease of young stock and that second and third abortions are less common. If we control the data by the bacteriological results obtained, we find that relatively few cows are subject to disease of the fetal membranes due to *Bacillus abortus* twice. Abortions do occur in succession but they are due chiefly to causes other than *Bacillus abortus*. In illustration of this fact the following consecutive abortions studied bacteriologically are noteworthy. The individual abortions have been given case numbers; each pair of numbers therefore refers to one and the same cow.

Case 3.—Cow aborted Feb. 23, 1916. Had six or seven normal calves before. Fetus contained within membranes, about 6 to 7 inches long. Fetus has undergone autolytic changes. Only head, limbs, and ribs recognizable. The entire mass has a reddish, translucent, gelatinous appearance. Guinea pigs inoculated with suspensions of ground cotyledons remained well. Cultures from spleens negative. This case cannot be considered as having been examined thoroughly bacteriologically, but *B. abortus* was most probably absent.

Case 159.—The same cow aborted Nov. 7, 1917, after having calved normally Feb. 12, 1917. Fetus 16¾ inches long. Some of the placenta discharged. Fetus had been dead *in utero*, probably for some time. From this case spirilla were isolated. Tests for *B. abortus* negative.

Case 146.—Holstein heifer aborted on pasture Oct. 22, 1917. Fetus could not be found. A portion of placenta removed next day. All of twelve guinea pigs inoculated from this for another experiment were found infected with *B. abortus* when chloroformed. *B. abortus* also identified in sections of placenta.

Case 259.—The same cow aborted July 18, 1918. Fetus 20 inches long. General subcutaneous, blood-tinted edema. Some of abdominal organs eaten out by some animal. Thorax full of blood-tinted fluid. Fetus probably dead some time as autolytic changes were prominent. Cultures from intact organs—lungs, liver, and kidneys—show large numbers of colonies of a bacillus diagnosed as *B. pyogenes*. Guinea pig tests of lung and meconium negative.

Case 30.—Calved Apr. 3, 1917, but calf lived 2 hours only. Probably 2 weeks premature. This cow had calved normally in 1914 and 1916. *B. abortus* was isolated from the uninflated lungs both in cultures and through guinea pigs.

Case 211.—The same cow aborted Mar. 13, 1918. Placenta retained. Fetus 23 inches long; has undergone more or less maceration *in utero*. Cuticle peels off readily. Large serous cavities filled with blood-tinted fluid. Edema of subcutis and muscular tissue, blood-stained. Cultures from this case remained sterile. Guinea pig tests also negative.

Case 19.—Cow gave birth to twin calves Mar. 13, 1917 (second pregnancy). Both very weak, unable to stand and too weak to drink. One calf died Mar. 15, of scours. Extensive fatty degeneration of liver. Many collapsed lobules throughout left lung and in ventral lobe of right lung. Two guinea pigs inoculated with suspension of ground lung tissue in salt solution became infected with *B. abortus*.

Case 290.—The same cow on Nov. 19, 1918, gave birth to a fetus 24 inches long. Placenta retained. Guinea pig inoculations negative as to *B. abortus*. Spirilla in pure culture isolated from fourth stomach and colon contents, from liver, spleen, kidney, and lungs.

Case 21.—Heifer aborted Mar. 20, 1917. Fetus about 25 inches long. General subcutaneous edema. Large serous cavities filled with blood-tinted fluid. From the placenta *B. abortus* was isolated through a guinea pig.

Case 301.—The same heifer was bred four times and gave birth on Dec. 11, 1918, to a calf apparently normal. Placenta retained; cow was discharging from vagina. A swab containing such discharge washed out in salt solution and the latter injected into two guinea pigs. From both *B. abortus* was subsequently isolated. The calf did not do well and was killed when 7 days old. One umbilical artery close to umbilicus was involved in suppuration. Multiple foci of collapse in both ventral lobes. From a guinea pig inoculated therewith *B. abortus* was isolated.

Case 246.—Fetus 7 to 8 inches long found enclosed in membranes on the morning of May 28, 1918. Autolytic changes far advanced. No odor of bacterial decomposition. Spirilla were isolated in pure culture from the lungs and liver. No other bacteria found in the fetus. Guinea pigs inoculated with material from the same organs were normal when chloroformed after 6 weeks. This cow was purchased Sept. 2, 1917, and aborted in Nov. of the same year. The fetus from this abortion was not received for study.

Case 350.—The same cow aborted a third time Apr. 30, 1919. Fetus, due June 4, alive at birth and able to stand. Weighs 57 lbs. Killed about 4 hours after birth. Autopsy shows that no food had been taken but the rumen contained besides the fetal fluid much froth from swallowed saliva. No abnormalities were detected. The placenta was retained and films from a swab inserted into uterus showed groups of bacteria resembling *B. abortus*. Cultures from the stomachs were impure mixtures, as might have been expected, but those from colon and rectum contained only *B. abortus*. A guinea pig inoculated with salt solution suspension from the swab, chloroformed after 7 weeks, was found with the lesions of *B. abortus*.

These six pairs of consecutive abortions indicate in general an acquired immunity to *Bacillus abortus*. In three pairs the first abortion was associated with *Bacillus abortus*, the second with negative cul-

tures, *Bacillus pyogenes*, and spirilla respectively. In one pair both abortions were associated with *Bacillus abortus*. In one pair *Bacillus abortus* was not detected either time. The first abortion may or may not have been associated with spirilla, since the condition of the material (advanced autolysis) did not warrant cultures, except from the spleen. These were negative. Later studies indicated that the spleen is rarely invaded by spirilla. The last case is exceptional. Of the first abortion there is no record. It may or may not have been due to *Bacillus abortus*. The second abortion was associated with spirilla. The third, associated with *Bacillus abortus*, may have been due to reinfection from the udder following loss of immunity acquired on account of the first abortion.

Were it not for agents other than *Bacillus abortus*, such as *Vibrio fetus*, and to a far less degree to miscellaneous septic and pyogenic organisms, and to unknown, non-bacterial agencies, second and later abortions by the same cow would be relatively rare. It therefore becomes necessary in the future to distinguish between the tendency of any given cow to abort repeatedly and the relation of *Bacillus abortus* to such repeated abortions. If abortions are due to a variety of infectious and non-infectious agencies, a better knowledge of abortion can only be reached by a patient, thorough study of series of individual cases, followed by the necessary experimental tests in order that these agencies may be assigned their proper place. A further analysis of this material will be found elsewhere.[5] It is there shown that the udders of a relatively high percentage of cows become infected with *Bacillus abortus* probably during the first abortion disease. Cows while carrying *Bacillus abortus* in the udder may give birth to normal calves, or to fetuses infected with spirilla, or to sterile fetuses. Probably the udder becomes a protecting reservoir of immune bodies towards *Bacillus abortus*.

SUMMARY.

In a large herd of dairy cattle and young stock the bacteriological examination of 109 cases of abortion which included a relatively thorough study of the fetus and a study of the membranes, or swabs from

[5] Smillie, E. W., Little, R. B., and Florence, L., *J. Exp. Med.*, 1919, xxx, 341.

the uterus whenever obtainable, gave the following results. 62, or 57 per cent, were associated with *Bacillus abortus*. 26, or 23.8 per cent, were associated with spirilla. 2, or 1.8 per cent, were associated with *Bacillus pyogenes*. 19, or 17.4 per cent, were either sterile or else the digestive and respiratory tracts had been invaded during or after birth with miscellaneous bacteria. *Bacillus abortus* was absent according to cultures and animal tests.

Such a relatively large proportion of cases of abortion without *Bacillus abortus* as the inciting agent is noteworthy. In general *Bacillus abortus* was associated with first pregnancies. Its presence diminished rapidly in frequency in later pregnancies. Assuming in a general way that purchased cows coming from small herds were free from any immunity and that their first pregnancy in the new herd is equivalent to that of a native heifer and may be counted as the first, we have *Bacillus abortus* associated with the first pregnancy in 42, with the second in 14, with the third in 5, and with the fourth in 1. Spirilla were distributed as follows: (*a*) in purchased cows, first pregnancy, 6; second pregnancy, 9; third pregnancy, 5; and fourth pregnancy, 3; (*b*) in native cows, first pregnancy, 0; third pregnancy, 1; sixth pregnancy, 1; and eighth pregnancy, 1. The relation of infection with spirilla to acquired immunity is not clear and more data from large herds are needed to define both etiological and immunological bearings of the spirilla.

Thus far spirilla have not been encountered in native heifers of the herd giving birth the first time. A tentative explanation to be offered is that the young stock is kept segregated from the older and purchased cows until shortly before calving. The occasional discharge of a fetus among the young stock in pasture tends to keep up the disease due to *Bacillus abortus*. Later on association with older cows brings about infection with spirilla (*Vibrio fetus*) and more rarely with other possible agencies of fetal disease. On the other hand, abortions may occur among the pastured stock from time to time and remain unrecognized. Not until both groups of animals are subjected to the same daily scrutiny will it be possible to affirm that abortion associated with spirilla does or does not occur among young stock.

AN INTERPRETATION OF THE AGGLUTINATION REACTION TO BACILLUS ABORTUS IN 75 CASES OF BOVINE ABORTION BACTERIOLOGICALLY CONTROLLED.

By ERNEST W. SMILLIE, D.V.M., RALPH B. LITTLE, V.M.D., and LAURA FLORENCE.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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In the following pages are given the results of a study of the agglutination test in 75 cases of abortion of which bacteriological data were at hand. Concerning these data and the methods employed in obtaining the results here used to interpret our findings, the reader is referred to Dr. Smith's paper.[1] Although bacteriological data on a much larger number of cases were available, yet in many instances the cows were removed from the herd before samples of blood could be taken. While this work was in progress certain discrepancies came to the surface and the search for a focus of infection with *Bacillus abortus* in the udder was deemed essential by Dr. Smith. Accordingly the milk from certain selected cases was brought into the investigation.

The existing literature on the agglutination test is voluminous and the work in most cases of high quality, yet there is lacking the bacteriological basis of a series of spontaneous cases for a more accurate interpretation of the test. The fact that Smith[1] found in 109 cases of abortion or premature expulsion of a living calf 46 not associated with *B. abortus* shows that our information in regard to the significance of the agglutination test has been built on somewhat insecure foundations.

Most writers have confined their attention to a comparison between the agglutination and the complement fixation test. This was done by Mohler and

[1] Smith, T., *J. Exp. Med.*, 1919, xxx, 325.

Traum,[2] Moore and Fitch,[3] Larson,[4] Rettger and White,[5] Wall,[6] and Zwick and Zeller.[7] Others have contented themselves with associating the serological reactions with abortion pure and simple. Wall,[6] Grinsted,[8] and Moore and Fitch[3] studied among other things the rise and decline of the agglutination curve. Since none of these researches bears directly upon the substance of this communication any detailed analysis would be out of place.

Methods.

Agglutination Technique.—The obtaining of blood samples from cattle is frequently difficult, since it depends on the way the animals are stabled and their degree of wildness or domestication. Those from which samples were collected for the present investigation were in the main milking cows. They were secured in stanchions, facing on a wide alleyway. A rope halter was placed on the head of the animal and the neck and head were extended by fastening the rope, so attached, to an iron rail supporting stanchions on the other side of the alley, and tightening the rope gradually until the jugular furrows were fully exposed. Strong bull nose forceps were then put on the anterior part of the nasal septum to hold the attention of the animal, and also to steady the head. One assistant can handle both the rope and bull nose forceps.

When the animal was well under control the field of operation was thoroughly wiped off with 95 per cent alcohol. Pressure was applied over the jugular vein at the posterior end of the furrow to make the vein prominent and a $2\frac{1}{2}$ inch needle No. 14 was inserted. 25 to 30 cc. of blood were collected in a sterile 50 cc. centrifuge tube. A separate, sterile needle was used for each sample. The samples were stored at room temperature until the clot had formed, after which it was separated from the sides of the tube with a heavy sterile wire.

[2] Mohler, J. R., and Traum, J., *U. S. Dept. Agric., Bureau of Animal Industry, Bull. 216*, 1913, 147.

[3] Moore, V. A., and Fitch, C. P., *Rep. N. Y. State Vet. College*, 1912–13, 82.

[4] Larson, W. P., *J. Infect. Dis.*, 1912, x, 178.

[5] Rettger, L. F., and White, G. C., *Storrs Agric. Exp. Station, Bull. 93*, 1918.

[6] Wall, S., *Z. Infektionskrankh. Haustiere*, 1911, x, 23, 132.

[7] Zwick and Zeller, *Arb. k. Gsndhtsamte.*, 1912, xliii, 1.

[8] Grinsted, P., Maanedsskrift f. Dyrlaeger, 1909, xxi, Review in *Berl. tierärztl. Woch.*, 1909, xxv, 831.

The blood samples were then placed in the refrigerator for 12 hours to allow the clot to contract completely. Each tube was centrifuged for 10 minutes, to throw the clot and free corpuscles to the bottom. With sterile bulb pipettes the clear serum was transferred from the centrifuge tubes to sterile, corked test-tubes for storage. No disinfectant was added to the serum as it was found unnecessary if each step of the procedure was carried out carefully.

The antigen used was always prepared fresh as needed. A stock culture of *Bacillus abortus* recovered early in this work by Dr. Smith was used. A 48 hour slant agar culture was washed off with 2 cc. of normal saline solution and the suspension transferred to an agar surface within a rectangular 16 ounce bottle. The bottle was sealed and incubated for 72 hours at 37°C., then washed off with 20 cc. of normal saline solution, and the suspension transferred to a sterile bottle. This very dense suspension was standardized by reducing the density with normal saline solution to a point at which the translucency equals that of a 24 hour bouillon culture of the typhoid bacillus. It was then ready for use.

In order to simplify the technique as much as possible, reduce the amount of glassware used, and safeguard the operations by using the same amounts of fluids in the series of tubes in which clumping was to be observed the following method was used throughout. Eight stock dilutions of serum were used as a starting-point. To the first tube 4 cc. of salt solution and 1 cc. of undiluted serum were added to make a stock dilution of 1:5. Into each of the other tubes $2\frac{1}{2}$ cc. of salt solution were placed. Then $2\frac{1}{2}$ cc. of the 1:5 serum dilution were added to the second tube, the mixture was drawn up several times, and $2\frac{1}{2}$ cc. of this were transferred to a third tube, and so on successively to the last. Each tube, except the final dilution, then contained $2\frac{1}{2}$ cc. of salt solution and $2\frac{1}{2}$ cc. of serum dilution, making 5 cc. in all and furnishing dilutions of serum beginning with 1:5 and ending in 1:640. To eight fresh tubes $\frac{1}{2}$ cc. of each serum dilution was now added, beginning with the highest dilution and using the same pipette up to the most concentrated solution. Finally $\frac{1}{2}$ cc. of the suspension of bacteria was added, making a series of serum dilutions beginning with 1:10 as shown in Table I. Particles in the culture suspension, such as bits of agar and clumps of bacteria, were

removed by withdrawing the suspension with a pipette through a piece of sterile cotton dropped into the stock suspension.

The distance or interval between the last tube of the series which was completely cleared by the clumping and sedimentation and the tube which showed the agglutination limit varied somewhat from case to case, as shown in Table II, which contains three illustrative examples taken from the protocols.

TABLE I.

Serum Dilution. (*First Series of Tubes.*)

Tube No.	1	2	3	4	5	6	7	8
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
Salt solution added	4	2½	2½	2½	2½	2½	2½	2½
Undiluted serum to first tube	1							
Diluted serum to other tubes in succession		→2½	→2½	→2½	→2½	→2½	→2½	→2½
Dilutions	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640.

Final Dilution. (*Second Series of Tubes.*)

Serum dilutions	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640
	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
Amount of serum dilution added to each tube	½	½	½	½	½	½	½	½
Culture suspension	½	½	½	½	½	½	½	½
Final dilution	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280

This method of using at least eight final dilutions served to reveal any possible errors and control any so called paradoxical reactions. Unless the series showed a definite regular decline in agglutinins, it was done over again. By this means most paradoxical reactions were traced to some irregularity or accidental error in manipulation. Table II illustrates the protocols. Readings were made after 3 to 4 hours in the incubator, followed by refrigeration over night.

The final agglutination titer adopted was shown by that tube in which there was some definite clumping as indicated in the suspension

or amount of deposit when compared with the next lower and negative tube and the culture control. In Table II the limit is shown as 1:640 in Case 31. Nos. 19 and 101 require further dilutions to obtain the precise limit. When clumping occurred in the control the series was rejected and a fresh one prepared. The figures given in Tables III to V represent, therefore, the agglutination limit under the conditions of the test as described above.

Test for Bacillus abortus in the Udder.—The milk was collected directly into sterile glass jars during the latter part of milking. The samples averaged about 650 cc. At the laboratory the milk was transferred to sterile separatory funnels and kept over night at

TABLE II.

Illustration of the Range between Complete Sedimentation and Slight or No Clumping.

Case No.	Dilutions.								Control.
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	
31	C.*	C.	C.	C.	++ ++	++ +	++	−	−
101	"	"	"	"	C.	++ ++	++ ++	++	−
19	"	"	"	"	"	C.	C.	++ +	−

* C. indicates complete clearing of the fluid, ++++ slight clouding plus heavy deposit, and so on to −, which means no trace of clumping.

about 4–5°C. The next morning the milk was withdrawn from the funnel, leaving the cream, distributed into large centrifuge bottles, and centrifuged for 20 minutes at a speed of about 1,500 revolutions per minute. Any cream remaining was removed with a section lifter, some of the top milk withdrawn with a pipette or syphon, and the remaining milk and sediment were well mixed and distributed into smaller centrifuge tubes and centrifuged a second time for 15 minutes at a speed of about 1,400 revolutions per minute. The top milk was again withdrawn, the remaining milk and sediment were well mixed, and each sample was injected intraperitoneally into three guinea pigs. Each animal received 5 to 7 cc. Films of the milk

fresh and fixed in absolute alcohol and ether and stained with alkaline methylene blue or Giemsa's stain were examined to determine in general the character of the sediment injected. After a period of 6 to 8 weeks the guinea pigs were chloroformed and three cultures made from each animal by tearing out bits of spleen tissue and transferring to agar slants whether lesions were present or not. All cultures were sealed.

Agglutination Titer towards Bacillus abortus when the Fetus Contains Only Spirilla (Vibrio fetus).

For more detailed information concerning the bacteriological data used in the tables to follow, the reader is referred to earlier papers by Smith.[1,9,10] Certain unpublished data were also furnished by him in individual cases to be discussed later.

In Table III are given data pertaining to nineteen cases in which spirilla were found and agglutination tests made. The remaining seven cases of the same group are omitted because blood samples were not obtained.

Taking an agglutination titer up to and including 1:40 as negative as to the presence of *Bacillus abortus*, we find that in eleven cases the agglutination titer agrees with the bacteriological findings. Of these eleven cases, the milk of three was examined for *Bacillus abortus* and found free. In two cases (Nos. 256 and 290) a maximum titer of 1:1,280 for *Bacillus abortus* was associated with the presence of this bacillus in the milk. This high titer is thus accounted for. The first four cases in the table have titers which indicate that the animals were infected with *Bacillus abortus* at some earlier date, since in three of these the milk tests were negative. The fourth was not examined. Of these No. 246 is of interest. This cow aborted May 28, 1918, with spirilla in the fetus. The fetus was small, only $7\frac{1}{2}$ inches long. On September 5 and 12, blood samples registered a titer of 1:160. On February 4, 1919, a sample of milk proved free from *Bacillus abortus*. On April 30, the cow aborted again (now Case 350). *Bacillus abortus* was found in the fetus. The titer of the

[9] Smith, T., *J. Exp. Med.*, 1918, xxviii, 701.
[10] Smith, T., *J. Exp. Med.*, 1919, xxx, 313.

blood on May 16 was 1:640. Evidently a fresh infection with *Bacillus abortus* had taken place since the previous abortion. In No. 159 there was a decline in the agglutination titer following the birth of a normal calf, asphyxiated at birth. Case 251 is the only one in the table whose titer cannot be satisfactorily explained with the data on hand. This cow aborted June 10, 1918, with positive spirilla cultures. The results were negative as to *Bacillus abortus*. On September 5, the blood titer for *Bacillus abortus* was 1:640. Two milk tests with samples, collected November 20, 1918, and March 4, 1919, were both negative. The only explanation that suggests itself is that there was associated *Bacillus abortus* with the spirilla in the fetal membranes but not in the fetus in the June abortion. The absence of any tests with uterine exudate immediately after abortion leaves this interpretation open. It is also possible that *Bacillus abortus* was in the milk in very small numbers. This case is thus the only one in the table in which the serological test does not harmonize with the bacteriological data.

One of the most instructive cases in Table III is Cow 290. The peculiarity of the agglutination titer makes a more detailed history of this case of interest. This cow gave birth to twins March 13, 1917. Both were unable to drink and too weak to stand. One was brought to the laboratory dead March 15. From Dr. Smith's notes on this case we find that there was marked fatty degeneration of the liver and extreme congestion of the medullary zone of the kidneys. The mucosa of the fourth stomach was covered with a thick viscid layer of mucus. In the lungs there were many lobules and groups of lobules collapsed. The postmortem changes made examination of the small intestines useless but the calf probably died of what is usually known as scours. Histological examination of fixed and hardened tissues showed bronchopneumonic collections of cells resembling polynuclear leucocytes, general vacuolation of liver cells, and intense congestion of the medulla of the kidneys approaching hemorrhage. Pieces of the collapsed lung tissue ground and inoculated into two guinea pigs produced the abortion disease in both cases. From these *Bacillus abortus* was isolated.

A dead fetus was discharged November 19, 1918, 24 inches long (No. 290). Spirilla were isolated in pure culture from the fourth

TABLE III.

Agglutination Test for B. abortus in Cases of Abortion Associated with Spirilla.

Case No.	Length of fetus.	Native or purchased cow.	Date of abortion.	No. of pregnancy.	Date of blood sample.	Agglutination titer (limit).	Date of milk sample.	Test for *B. abortus* in milk.	Remarks.
	in.								
159	16¾	Purchased.	Dec. 17, 1917	3	Aug. 29, 1918	1:160			Preceding pregnancy also terminated in abortion. Etiology in doubt. Asphyxiated at birth.
(324)	Normal calf.		Feb. 12, 1919	4	Mar. 24, 1919	1:20			
192	36	Purchased.	Jan. 23, 1918	3	Aug. 29, 1918	1:80	Dec. 17, 1918	−	
213	21	"	Mar. 18, 1918	4	" 29, 1918	1:160	" 17, 1918	−	
246	7½	"	May 28, 1918	2	Sept. 5, 1918	1:160	Feb. 4, 1919	−	
					" 12, 1918	1:160			
(350)	Calf alive.		Apr. 30, 1919	3	May 16, 1919	1:640			*B. abortus* found in calf.
251	17½	Purchased.	June 10, 1918	2	Sept. 5, 1918	1:640	Nov. 20, 1918	−	
							Mar. 4, 1919	−	
256	19	"	" 18, 1918	4	Oct. 3, 1918	1:1,280	Nov. 27, 1918	+	
258	27	"	" 29, 1918	2	" 3, 1918	1:20	Feb. 11, 1919	−	
267	23	"	Aug. 12, 1918	3	" 3, 1918	1:40			
269	20	"	Nov. 18, 1918	2	Nov. 21, 1918	1:20	Jan. 7, 1919	−	
					" 28, 1918	1:20			

290	24	Native.	Nov. 19, 1918	3	Nov. 14, 1918 " 21, 1918 " 28, 1918 Dec. 11, 1918	1:10 1:1,280 1:1,280 1:1,280	Nov. 27, 1918	+	Preceding pregnancy (twins) associated with *B. abortus* (Mar. 13, 1917). Agglutination titer still 1:1,280 Apr. 4, 1919.
308	31½	Purchased.	Dec. 30, 1918	1	Jan. 2, 1919	1:40			
317	25	"	Jan. 27, 1919	2	Feb. 7, 1919	1:10	Mar. 21, 1919	−	
318	26	"	" 27, 1919	2	" 7, 1919	1:10			
331	8	Native.	Mar. 15, 1919	6	Mar. 24, 1919	1:10			
333	26	Purchased.	" 27, 1919	1	" 29, 1919	1:10			
339	35½	"	Apr. 9, 1919	3	" 29, 1919	1:40			
351	28	"	May 5, 1919	1	May 15, 1919 June 2, 1919	1:20 1:160			Fetus contained *B. abortus* also.
356	26½	"	" 23, 1919	1	" 2, 1919	1:80			
357	21¾	Native.	" 24, 1919	8	" 2, 1919	1:10			

stomach, large intestine, lungs, kidney, spleen, and liver. Guinea pigs inoculated with lung tissue, meconium, fourth stomach contents, and suspensions of exudate from the vagina obtained with a swab November 18 were normal when killed after 7 weeks.

On November 14, 1918, 5 days before abortion, the agglutination titer was only 1:10. 7 days later and 2 days after discharge of the fetus it was 1:1,280. The same high titer was found on November 28 and December 11. Milk drawn November 27 was found infected with *Bacillus abortus*. On April 4, 1919, the titer was still 1:1,280.

The main facts in the history of this cow are: Twins March 13, 1917, one of which carried *Bacillus abortus* in lungs. Discharge of fetus November 19, 1918, containing spirilla but not *Bacillus abortus*. Agglutination titer towards *Bacillus abortus* jumped from 1:10, 5 days before, to 1:1,280, 2 days after abortion. We have thus far been unable to find a second case with conditions paralleling this and hence are unable to present a confirmation of this somewhat unique case. Possible errors in collecting samples and in assigning the fetus to the right dam can be eliminated. The sudden rise in agglutinins may be tentatively ascribed to a rapid absorption of *Bacillus abortus* or some antigen from the udder into the circulation during discharge of the fetus. The mechanism for producing agglutinins towards *Bacillus abortus* having been established since the preceding calving, it acted promptly on stimulation by discharging large amounts of agglutinin into the blood.

No. 356 has a suspicious titer probably due to earlier *Bacillus abortus* infection. No. 351, a double infection, is discussed farther on.

In Table IV are brought together such cases of abortion as were characterized by sterile fetuses or else by miscellaneous infection of the digestive and respiratory tracts. Bacteriological details will be found elsewhere.[1]

Of the eleven cases, eight show a titer of 1:20 or lower. One (No. 211) with a high titer of 1:640 is explained by an earlier *Bacillus abortus* infection and by a demonstration of *Bacillus abortus* in the milk. The first sample of milk was negative, the second positive. In another (No. 259) with a titer of 1:160 this may be regarded as a remnant of the preceding pregnancy which terminated in abortion associated with *Bacillus abortus*. The same may be true of a third

TABLE IV.

Agglutination Titer in Cases of Abortion with Sterile Fetus or Miscellaneous Infection.

Case No.	Length of fetus.	Native or purchased cow.	Date of abortion.	No. of pregnancy.	Date of blood sample.	Agglutination titer (limit).	Date of milk sample.	Test for *B. abortus* in milk.	Remarks.
	in.		*1917*		*1918*		*1919*		
103	4	Native.	June 24	3	Oct. 30	1:10			Normal births Aug. 8, 1918, and Mar. 26, 1919.
122	24	Purchased.	July 31	1	Nov. 14	0			Normal calf Nov. 16, 1918.
126	(75 lbs.)	"	Aug. 15	2	" 14	1:20			" " " 19, 1918.
143	(35 ")	"	Oct. 11	1	Oct. 30	1:20	Jan. 10	−	" " Oct. 17, 1918.
			1918						
201	34	"	Feb. 12	2	" 9	1:10	" 10	−	
211	23	Native.	Mar. 13	4	Sept. 27	1:640	" 10	−	Preceding pregnancy terminated Apr. 4, 1917, by *B. abortus* infection.
					1919				
					Apr. 26	1:1,280	Apr. 11	+	Normal calf Apr. 4, 1919.
					June 3	1:2,560			
					1918				
259	20	"	July 18	2	Oct. 3	1:160	Jan. 2	−	Preceding pregnancy infected with *B. abortus*; present one with *B. pyogenes*.
262	29	Purchased.	" 28	1	" 3	1:20			
279	8	Native.	Sept. 27	2	" 3	1:320			Aborted Jan. 20, 1918. No bacteriological data.
291	32	"	Nov. 19	4	Nov. 28	1:10	Jan. 2	−	Infection with *B. pyogenes*.
300	35	Purchased.	Dec. 7	6	Dec. 11	1:20			

TABLE V.

Agglutination Reaction of Cows, Native and Purchased, from Which B. abortus Was Isolated.

Case No.	Length of fetus.	Native or purchased cow.	Date of abortion.	No. of pregnancy.	Date of blood sample.	Agglutination titer (limit).	Date of milk sample.	Test for *B. abortus* in milk.	Remarks.
	in.		*1917*		*1918*		*1918*		
17	27	Native.	Mar. 9	1	Nov. 7	1:40			Normal calf Apr. 14, 1918.
26		"	" 27	1	" 21	1:20			" calves Jan. 17, 1918, and Mar. 22, 1919.
31	27	Purchased.	Apr. 5	1	" 28	1:640	Dec. 17 *1919*	+	Normal calf Oct. 3, 1918.
33	26	Native.	" 6	1	" 21	1:640	Feb. 18	+	" " June 8, 1918.
34*	Living calf.	"	Feb. 23		" 21	1:320	" 20	+	" calves Feb. 23, 1917, and Mar. 3, 1918. Cow aborted Nov. 10, 1918. No bacteriological examination.
41	Nearly mature.	"	Apr. 22	1	" 14	1:40	" 20	+	Normal calf Oct. 17, 1918.
43	(8 mos.)	"	" 23	1	" 21	1:640	" 20	+	" " Apr. 23, 1918.
86	21½	"	May 16	1	" 14	0			" " Oct. 25, 1918.
89	Alive.	"	" 16	1	" 21	1:40			" " Aug. 6, 1918.
91	30	Purchased.	" 5	1	" 21	1:80			" " " 1, 1918.
101*	Normal calf.	Native.	June 28	1	Oct. 30	1:1,280	Feb. 28	+	Placenta appears normal. Normal calf June 9, 1918.
128	Calf dies (scours).	Purchased.	Aug. 17	1	" 20	1:10			*B. abortus* isolated from calf's intestine. Normal calf Sept. 26, 1918.
134	Calf alive.	Native.	Sept. 4	1	Nov. 14	1:320	Feb. 18	+	*B. abortus* obtained from placenta. Normal calf Nov. 8, 1918.

144*	Normal calf.	Purchased.	Oct. 8	1	Oct. 30	1:640	Mar. 4	+	Normal calf Mar. 21, 1919.
164	Alive.	Native.	Nov. 20	1	" 9	1:320	" 4	+	7 wks. premature.
					1919				
188*	Calf alive.	Purchased.	Dec. 31	1	Jan. 2	1:1,280	Feb. 26	−	
			1918		*1918*				
200	21	Native.	Feb. 12	1	Oct. 17	1:640	" 26	+	Calf very sick with scours when killed.
203	Calf alive.	"	" 15	1	Sept. 27	1:640			
206	28	"	" 24	1	" 27	1:160			
210	Calf alive.	"	Mar. 6	1	" 27	1:40			
214	" "	Purchased.	" 15	1	" 27	1:320			
215	" "	Native.	" 11	1	" 18	1:1,280			
							1918		
220*	" "	"	" 30	1	" 18	1:640	Nov. 12	+	Multiple staphylococcus abscesses in lung.
222	" "	Purchased.	Apr. 11	1	" 18	1:1,280			
224	" "	Native.	" 12	1	" 18	1:640			
226	" "	"	" 12	2	" 12	1:1,280			
227	" "	"	" 15	1	" 12	1:640			
253	25	Purchased.	June 13	2	" 27	1:160			
					Oct. 3	1:320			
266	31	Native.	Aug. 5	3	Sept. 5	1:640	Nov. 20	+	
							1919		
270	29½	Purchased.	" 22	2	" 5	1:1,280	Jan. 11	−	
273	16	"	" 27	2	" 5	1:320			
278	14½	"	Sept. 19	1	Oct. 4	1:40			
281	30½	"	Oct. 4	1	" 9	1:160			
					1919				
285	28½	Native.	" 23	1	Jan. 2	1:640			
288*	Calf alive.	Purchased.	Nov. 17	1	" 2	1:1,280	Feb. 4	+	Calf has scours.

* Included because the milk was examined. *B. abortus* was not isolated from the calf.

TABLE V—*Concluded.*

Case No.	Length of fetus.	Native or purchased cow.	Date of abortion.	No. of pregnancy.	Date of blood sample.	Agglutination titer (limit).	Date of milk sample.	Test for *B. abortus* in milk.	Remarks.
	in.		*1918*		*1918*		*1918*		
298	34	Native.	Dec. 2	1	Dec. 11	1:80			
301	Calf alive.	"	" 11	2	Nov. 28	1:1,280	Dec. 10	+	*B. abortus* isolated from uterine exudate. *B. abortus* found in fetus expelled Mar. 20, 1917.
			1919		*1919*				
309	(58 lbs.)	Purchased.	Jan. 2	1	Jan. 8	1:320			
311	(38 ")	"	" 7	1	" 16	1:640			
313	29	Native.	" 12	4	" 23	1:640			No record of former abortions.
316	22¼	"	" 18	2	" 18	1:1,280			
328	31	"	Mar. 9	2	Mar. 24	1:640			
329	30	Purchased.	" 11	1	" 24	1:640			
350	Calf alive.	"	Apr. 30	3	May 16	1:640			Aborted in preceding pregnancy.
351	28	"	May 5	1	" 15	1:20			Fetus contains both spirilla and *B. abortus*.
					June 2	1:160			

case (No. 279). Unfortunately a milk sample from this cow was not obtainable.

In Table V all cases of abortion associated with *Bacillus abortus* in the fetus or membranes or in the udder are brought together. The agglutination limits as given easily bring the cases into several categories. Taking those cases in which the serum clumps *Bacillus abortus* in dilutions of 1:20 and 1:40, but not up to 1:80, we find, out of 44, 8 in this class. The low titer in certain cases (Nos. 17, 26, 41, 86, 89, and 128) may be referred to the long period between abortion and the blood test, ranging from 14 to 20 months. In No. 210, the low titer of 1:40 may be due to a mild, possibly quite recent infection of the fetal membranes. The calf was vigorous and apparently in normal condition. No. 278 with a titer of 1:40 discharged a macerated fetus about $3\frac{1}{2}$ months old. Here also the infection may have been too recent to stimulate antibodies.

No. 351 is of interest since both *Vibrio fetus* and *Bacillus abortus* were isolated from the fetus. The dam was well along ($6\frac{1}{2}$ to 7 months) in pregnancy. *Bacillus abortus* infection may have been superinduced late on the spirillum infection, since the agglutination titer for *Bacillus abortus* rose after abortion.

There are but two cases with a titer of 1:80. In one (No. 91) this was determined 18 months after abortion and may have fallen from a higher level. The second (No. 298) cannot be explained in the same way since the blood test was made 9 days after abortion. In this case, however, the fetus was large and the duration of pregnancy over 7 months. Here also the immune reaction of the dam may not have reached its maximum owing to late infection.

The two cases with a titer of 1:160 are probably explainable in much the same way as the two preceding ones. In one the blood test was made 5 days, in the other 7 months after abortion. Both fetuses were large.

Taking the next higher titer, 1:320, we find six on this level. Nos. 134 and 164 carried *Bacillus abortus* in the milk according to recent tests. How far back this milk infection dates cannot be determined. Most likely it started with the abortion indicated in the table. In the case of Nos. 214, 253, 273, and 309 the longest period between the discharge of fetus or calf and the agglutination test was less than 7 months.

The next highest titer, 1:640, was held by sixteen cases. In the two earliest cases recorded (Nos. 31 and 33) the blood test followed abortion by 19 months. The titer should have been low after such a long period but the udder was infected. In the next case (No. 144) the blood test followed abortion by nearly 13 months. In this case also the high titer is explainable by the udder infection. In the remaining thirteen cases the high titer is amply accounted for by the recent abortion. The milk, tested in only three of these, was found infected in all.

The highest recorded titer in this group, 1:1,280, was held by nine cases. The agglutination test in No. 101 is referable to the milk infection. No. 188 is an unexplained case. The test for *Bacillus abortus* in the milk was negative. A second sample is now being examined.[11] The other cases may be explained by the presence of *Bacillus abortus* in the fetus. No. 270 is of interest in that a high titer was present 14 days after abortion, although the sample of milk tested subsequently was free from infection.

SUMMARY.

The agglutination test when carried out so as to give the entire range of serum dilutions to the limit of clumping is a delicate test which reflects a variety of conditions involved in infection with *Bacillus abortus*. Among these conditions are its time relation to the act of abortion and the length of time the abortion bacilli live and multiply in the pregnant uterus. It is obvious that if a uterus be infected in the 8th month of pregnancy, the opportunity for agglutinins to accumulate are poorer than if the uterine infection lasts 3 or 4 months. The presence of *Bacillus abortus* in the udder determines in many cases the intensity of the reaction. No definite rules can therefore be formulated for the interpretation of the agglutination reaction quantitatively, since it is bound up with a complicated process varying from case to case. In the individual cow in general a titer of 1:40 or less may be regarded as indicating that the cow is not infected with *Bacillus abortus* at the time of the blood examination. It does not exclude former infections in the case of older cows, nor does it absolutely exclude very recent infection (Nos. 278 and 351).

[11] The second test of the milk of No. 188 was negative.

The highest titers, 1:640 and above, generally indicate recent infection and in the absence of recent premature births infection of the udder. Even when abortion has just occurred, it may be due to other agencies and the high titer maintained by a chronic infection of the udder dating from an earlier uterine infection with *Bacillus abortus*. Intermediate titers may indicate a gradual rise or decline of agglutinins preceding or following abortion without infection of the udder. They may also stand for a relatively high resistance or partial immunity of the cow.

In any herd a uniformly low titer (1:40 or less) in all animals may be regarded as indicating the entire absence of *Bacillus abortus*. A high titer in any one cow serves to indicate quite definitely the presence of infection in the herd. To determine more accurately the character of the infection in any individual cow there is needed in addition to the quantitative agglutination test a bacteriological study of the milk and of any prematurely discharged calf or fetus.

HYDROGEN ION CONCENTRATION OF CULTURES OF PNEUMOCOCCI OF THE DIFFERENT TYPES IN CARBOHYDRATE MEDIA.

By O. T. AVERY, M.D., and GLENN E. CULLEN, Ph.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

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Knowledge of the physiological activities and immunological characters of bacteria serves not merely the purposes of systematic classification, but contributes to a fuller understanding of the problems of infectious disease. The correlation of these apparently independent characters with pathogenicity and with the occurrence and distribution of recognizable types under a wide variety of environmental conditions is essential to the proper interpretation of the phenomena of infection.

Of the physiological characters of bacteria the fermentation of certain carbohydrates by some, and the inability of other closely related organisms to utilize the same substances, are relatively fixed characteristics of sufficient constancy to warrant their use as a basis for differentiation. In fact, within a given species these biochemical reactions are of considerable value in distinguishing type relationships. The application of this principle in the classification of the typhoid-dysentery group affords an illustration of the value of the biochemical method in determining the existence of certain varieties of these organisms and their relation to enteric infections. On the other hand, it has been possible in the case of pneumococcus to acquire knowledge of the occurrence of specific types and their relation to disease, chiefly by a study of the immunological characters of these organisms, since their biochemical activities are apparently possessed in common by all.

The measure of acid production by bacteria has been the determination of the amount of titrable acidity produced in a medium containing a known concentration of carbohydrate. This method is now recognized as inadequate. Recently the colorimetric determination

of the hydrogen ion concentration has been adapted to bacteriological requirements and affords an additional method for measuring this phase of bacterial metabolism. Moreover, in addition to the selective fermentation of certain substances by bacteria, the maximum acidity that can be tolerated by a given organism is apparently a biologic constant. Accordingly, the determination of the hydrogen ion concentration at which bacterial growth ceases has been utilized as a differential method. In the case of a culture growing in a medium containing an excess of fermentable substance this limiting reaction is spoken of as the final hydrogen ion concentration. This phenomenon has been utilized by Clark (1) and Clark and Lubs (2) in the differentiation of the colon-*aerogenes* group, and by Ayers (3), Ayers, Johnson, and Davis (4), and Avery and Cullen (5) in the recognition of differences between hemolytic streptococci of human and bovine origin.

The present paper presents the results, obtained under the experimental conditions defined, of a study of the influence of the concentration of dextrose on the final hydrogen ion concentration of broth cultures of pneumococcus, the rate of acid production, the optimum and limiting hydrogen ion concentration for initial growth of pneumococcus, the final hydrogen ion concentration of cultures of pneumococcus in carbohydrate media, and a comparison of these physiological functions with the specificity of the immunological type characters of these organisms. In addition, some information has been acquired concerning the factors governing growth of pneumococcus when reinoculated into filtrates of cultures of the same and different types.

EXPERIMENTAL.

Source of Material.—Thirty-nine strains of pneumococcus comprising representatives of the various immunological types were studied. In the majority of instances these cultures were isolated directly from the blood or sputum of patients suffering from lobar pneumonia. Some of the strains were tested immediately upon isolation, others after years of cultivation on artificial media. Most of the strains were pathogenic for white mice and had been passed through these animals to enhance virulence. Cultures used for inoculation of the test medium were grown for 18 hours at 37°C. in plain meat infusion broth of pH

7.8. The serological methods used for the determination of types of pneumococcus were the same as those already described (6).

Determination of Hydrogen Ion Concentration.—The colorimetric method of determining hydrogen ion concentration was chosen because of its convenience and simplicity. The principle and details of this method have been so thoroughly reviewed that it is unnecessary to repeat them here (7). In the present work the two principal sources of error, color of the medium and turbidity of the culture, have been avoided by first diluting the medium and then compensating for the color by Walpole's comparator method of superimposing the color of the medium upon that of the indicator.

The hydrogen ion concentrations are expressed in the customary manner as pH values; that is, the negative exponent to the base 10 of the normality.

Duplicate tubes containing 5 cc. of the medium or culture were diluted to 15 cc. with redistilled water, four drops of indicator solution were added to one tube, and the tube was compared in a comparator block with a standard solution containing exactly the same amount of indicator. The standard solutions of known hydrogen ion concentration were prepared for the range pH 8.6 to 5 from standard phosphates by Sörensen's technique, and for the range pH 5.8 to 4.6 from acetic acid-sodium acetate mixtures by Walpole's directions. The accuracy of these standard solutions was verified by the hydrogen electrode.

The indicators used were:

	per cent
pH 8.6–8.0, *o*-cresol sulfonephthalein	0.04
pH 8.0–6.8, phenolsulfonephthalein (phenol red)	0.04
pH 6.8–5.8, bromocresolsulfonephthalein, saturated water solution.	
pH 5.8–4.6, methyl red	0.04

Sterilization of the Medium Containing the Test Substance.—In studying fermentative activity of bacteria the manner in which the medium containing the test substance is sterilized is of first importance. If a sugar, such as sucrose, is added to the slightly alkaline broth and sterilized in an Arnold sterilizer or boiled for a long time, the sugar will be hydrolyzed. It seemed best, therefore, to sterilize by boiling a concentrated solution of the substance in water. Enough of this con-

centrated solution is then added to sterile broth to the desired dilution. 10 minutes in boiling water has sufficed in most cases to effect sterilization. When the medium has been handled many times, in adjusting to different reactions, further sterilization has been considered necessary. Additional sterilization is indicated in the protocol.

Influence of Sugar Concentration upon the Final Hydrogen Ion Concentration.—In studying the sugar-fermenting property of any organism it is, of course, essential to determine the influence of varying concentrations of sugar upon the final reaction. In order to do this for pneumococcus the following experiment was carried out.

To sugar-free broth of known hydrogen ion concentration sterile 20 per cent dextrose solutions (boiled 10 minutes) were added in sufficient amounts to make the desired concentration. The medium was incubated over night to test sterility, and 50 cc. portions were inoculated with 0.1 cc. of an 18 hour plain broth culture of pneumococcus. The hydrogen ion concentration of the medium was determined before the inoculation and after 24 and 48 hours incubation (Table I).

TABLE I.

Influence of the Concentration of Dextrose on the Final Hydrogen Ion Concentration of Pneumococcus Cultures.

50 cc. of sugar-free* broth, pH 7.5, inoculated with 0.1 cc. of 18 hour plain broth culture of Pneumococcus Type II (Strain F 149).

Dextrose.	Hydrogen ion concentration.	
	24 hrs.	48 hrs.
per cent	*pH*	*pH*
Broth control (uninoculated).	7.5	7.5
0	7.3	7.2
0.1	6.5	6.5
0.2	6.0	6.0
0.4	5.1	5.0
1.0	5.0	5.0
2.0	5.0	5.0
4.0	5.0	4.9

* In the preparation of sugar-free broth the meat infusion, before the addition of peptone, is fermented by *B. coli* for 18 to 24 hours.

It is evident that 0.4 per cent dextrose furnished sufficient acid to bring the medium to the limiting hydrogen ion concentration, but that excess of sugar up to at least 4 per cent has no influence upon the final reaction. 1 per cent, therefore, was chosen as sufficient for all routine fermentations. This agrees closely with a similar experiment with *Streptococcus hæmolyticus* (5).

Rate of Acid Production of Pneumococcus in 1 Per Cent Dextrose Broth.—It was to be expected that the rate of acid production would be dependent upon the size of the inoculum, but it was desirable to compare the rate curve in dextrose broth with that in plain broth. Moreover, it was necessary to determine the time required for the attainment of the final hydrogen ion concentration. In determining the rate a massive inoculum was used to bring the experiment within 1 day (Table II).

TABLE II.

Rate of Acid Production by Pneumococcus in Dextrose Broth.

100 cc. of sugar-free broth plus dextrose to 1 per cent, inoculated with 0.5 cc. of an 18 hour plain broth culture of pneumococcus, and incubated at 37°C.

Pneumococcus.	1 per cent dextrose broth. Initial hydrogen ion concentration.	Hydrogen ion concentration.					
		1½ hrs.	4 hrs.	6 hrs.	8 hrs.	9 hrs.	27 hrs.
	pH	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*
Type I	7.75	7.7	7.3	6.5	5.6	5.3	5.0
" II	7.9	7.9	7.5	6.8	5.7	5.3	4.8

No change in reaction of the dextrose broth occurred during the period of initial lag, then acid was produced at a rapid and constant rate until the final reaction was reached—in this case a pH 4.8 to 5.

With the usual inoculum employed in these experiments, 0.1 cc. of an 18 hour culture to 50 cc. of broth, the final hydrogen ion concentration is attained within 24 hours. An occasional culture will show a further increase in acidity of not more than 0.1 pH after 2 to 7 days incubation, but such changes are within the limits of experimental error. Table III furnishes an illustration of this fact. In a few instances in this experiment 18 hours was not sufficient time for the

attainment of the final reaction, but no apparent changes took place after 30 hours. As a routine, therefore, the cultures were read after 24 or 48 hours incubation at 37°C.

TABLE III.

Relation of the Type of Pneumococcus to the Final Hydrogen Ion Concentration in Sugar Broth.

75 cc. portions of 1 per cent sugar broth having an initial reaction of pH 7.8 were inoculated with 0.1 cc. each of an 18 hour plain broth culture of pneumococcus of different types.

Pneumococcus.		Hydrogen ion concentration.					
		Dextrose.			Lactose.		
Type.	Strain.	18 hrs.	30 hrs.	5 days.	18 hrs.	30 hrs.	5 days.
		pH	*pH*	*pH*	*pH*	*pH*	*pH*
I	F 55	5.1	5.1	5.1	5.1	5.1	5.1
I	146	5.0	5.1	5.0	5.0	5.0	5.0
II	46	4.9	5.0	5.0	5.0	5.0	5.0
II	F 149	5.2		5.1	5.1	5.1	5.1
IIa	J	5.8	5.0	5.0	5.3	4.9+	5.0
IIb	W	5.1	5.2	5.2	5.3	5.1	5.1
III	A 66	5.1	5.05	5.05	5.1	5.0	5.1
III	F 104	5.0	5.0	5.0	5.1	5.0	5.1
IV	L F	5.0	5.0	4.9	5.1	5.2	5.1
IV	L A	5.0	5.2	5.2	5.1	5.2	5.2

Relation of the Type of Pneumococcus to the Final Hydrogen Ion Concentration of Cultures in Sugar Broth.—In order to determine whether the differences in antigenic properties of the specific types of pneumococcus had any influence on the final reaction in sugar medium, two strains of each of the four types, as well as strains representing subgroups of Type II were inoculated into broth containing 1 per cent dextrose or lactose. It is evident from Table III that the final pH of all strains of pneumococcus was between pH 5.2 and 4.9, with the majority between pH 5.0 and 5.2. The differential characters as determined by immunological reactions were not evident from the final hydrogen ion concentrations. It is of significance that the final hydrogen ion concentration of cultures of pneumococcus is exactly the same as the final reaction of pathogenic hemolytic streptococci of

human type. This is also brought out in Table IV where the results of all the strains used in these and succeeding experiments are summarized by types. This table represents 54 determinations on 39 strains. Duplicate determinations in individual experiments are not included in this table.

TABLE IV.

Final Hydrogen Ion Concentration of Pneumococcus in Dextrose Broth. Summary of 54 Determinations on 39 Strains.*

Type I.		Type II.		Type III.		Type IV.	
Strain.	Hydrogen ion concentration.	Strain.	Hydrogen ion concentration.	Strain.	Hydrogen ion concentration.	Strain.	Hydrogen ion concentration.
	pH		*pH*		*pH*		*pH*
I	5.2	II	5.0	A 66	5.0	L F	5.0
I	5.0	II	4.9	A 66	5.1	L A	5.2
I	5.1	F 149	5.1	F 104	5.0	F 194	5.1
F 55	5.1	F 149	5.1	F 104	4.8	X 47	5.2
E 22	5.1	F 149	5.1	D 40	5.0	E 190	5.0
F 152	5.0	F 149	5.1	D EH	4.9	E 190	4.8
D 6	5.0	F 149	5.0	U	5.1	E 117	5.0
D 46	5.0	F 149	5.0	E 84	5.0	E 157	5.0
D 46	4.8	F 149	5.1	C 28	5.0	E 121	5.2
D 46	5.0	F 149	5.3	E 111	4.9	D 107	5.0
16,887	5.0	D 39	5.0	G	5.0	Sch.	4.9
16,867	5.0	IIa J	5.0	E 127	5.0		
F 169	5.0	IIb W	5.0	A	4.9		
		IIc A	5.0				
		IIc 50	5.0				
		II atypical, F 150	5.0				
		II " F 154	5.1				
Total 13		17		13		11	
Average.....	5.0		5.0		4.9		5.0

* Repetition of strains in the above table represents observations made in different experiments.

It is evident from the preceding experiment that with dextrose and lactose there is no type difference in acid production. A number of other carbohydrates fermentable by pneumococcus have been tested.[1]

[1] The substances used were either Kahlbaum's reagents or were those prepared by Mr. E. P. Clark of The Rockefeller Institute for Medical Research.

The media were prepared by placing solutions or suspensions of the substance in boiling water for 10 minutes, and adding the required amount to 75 cc. of media. The media were then heated in the Arnold sterilizer once for 20 minutes, and incubated to test sterility. They were inoculated with 0.1 cc. each of 18 hour plain broth cultures of pneumococcus representing the four types. Samples were removed for pH determinations after 48 hours and 7 days. Since 7 day readings are practically identical with 48 hour readings, only the latter are given (Table V).

TABLE V.

Final Hydrogen Ion Concentration of Pneumococcus in Various Carbohydrate Media.

75 cc. of sugar-free broth containing 1 per cent carbohydrate, initial pH 7.5, were inoculated with 0.1 cc. of an 18 hour plain broth culture and incubated 48 hours at 37°C.

Test substance.	Uninoculated control.	Pneumococcus type.			
		I (Strain F 152).	II (Strain F 149).	III (Strain F 104).	IV (Strain E 190).
	pH	*pH*	*pH*	*pH*	*pH*
Maltose	7.5	5.0	5.0	4.9	5.0
Saccharose	7.5	5.0	5.1	5.0	5.0
Lactose	7.5	5.0	5.0	5.0	4.9
Galactose	7.3	5.1	5.1	5.1	4.8
Raffinose	7.5	5.0	5.1	5.1	5.1
Dextrose	7.5	5.1	5.1	4.8	4.8
Inulin	7.5	5.1		5.1	5.1

From the results presented in Table V it is evident that in media containing sufficient fermentable carbohydrate, growth of pneumococcus continues until a final hydrogen ion concentration of about pH 5 is reached. Apparently this acidity is sufficient in itself to stop growth and the organisms die in the products of their own metabolism. Cultures of pneumococci with all the carbohydrates which were fermentable under the conditions used, namely maltose, saccharose, lactose, galactose, raffinose, dextrose, and inulin, gave identical results in the rate of reaction change and final hydrogen ion concentration (pH 5) attained. Further, the different immunological types of pneumococcus, in a limited number of strains studied, behave alike in fermenting these carbohydrates.

Optimum Hydrogen Ion Concentration for Growth of Pneumococcus.—Work previously reported from this laboratory (8) showed that the optimum hydrogen ion concentration for the growth of pneumococcus was about pH 7.8, and that the hydrogen ion concentrations within which growth could be initiated were between pH 7.0 and 8.3. It was desirable to determine whether the optimum pH in sugar broth was the same as that in plain broth and also to determine for dextrose broth the upper and lower hydrogen ion concentrations beyond which initial growth does not occur.

50 cc. portions of plain broth containing 0.2 per cent phosphate and the same broth containing 1 per cent dextrose were adjusted to the desired reactions by addition of either sodium hydroxide or hydrochloric acid. The media were placed in boiling water for 10 minutes, and incubated for 48 hours to test sterility. They were then inoculated with 0.25 cc. of an 18 hour culture of Pneumococcus Type I (Strain F 169). After 6 hours, estimation of growth and pH determinations were made, and a portion was autoclaved for determination of turbidity by the nephelometer method (Kober instrument). Essentially the same procedure was used as that described by Dernby and Avery (8), except that it seemed unnecessary to estimate the number of organisms per cubic centimeter, since it is in reality a function of the size of the inoculum. The readings on the nephelometer were expressed, therefore, as percentages of the heaviest growth (Table VI).

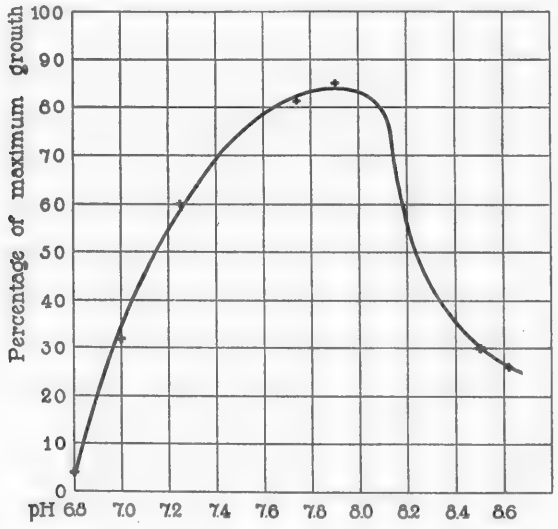
This method is entirely satisfactory with plain broth, and the results are in agreement with those of Dernby and Avery (8); it is beset with some difficulty, however, in the case of sugar broth. With plain broth the experiment may be continued to the maximum growth possible, but in sugar broth the readings must be made before acid precipitation takes place. Slight variations in reading and in initial lag are therefore emphasized. However, the curve constructed from the results was, with one exception, smooth (Text-fig. 1). The experiment was repeated with the same general result but with discrepancies in individual tubes. Another experiment was then planned, in which in addition to the turbidity method, pH determinations were made at regular intervals in order to give an idea of the time required to reach a given pH.

TABLE VI.

Optimum Hydrogen Ion Concentration for Growth of Pneumococcus.

50 cc. portions of plain broth and 1 per cent dextrose broth were adjusted to the indicated hydrogen ion concentration and inoculated with 0.25 cc. of Pneumococcus Type I (Strain F 169). Readings were made after 6 hours at 37°C.

Initial hydrogen ion concentration of media.	Plain broth.		Dextrose broth.	
	Turbidity.	Hydrogen ion concentration.	Turbidity.	Hydrogen ion concentration.
pH	*per cent*	*pH*	*per cent*	*pH*
8.4	23	8.1	80	7.5
8.3	70	7.5		
8.0	73	7.4	100	7.2
7.8	65	7.1	37	7.5
7.5	39	7.1	39	7.1
7.3		6.9	16	7.1
7.0		6.9		6.9
6.5				6.5
6.0				6.0
5.5				5.5



TEXT-FIG. 1. Effect of initial hydrogen ion concentration on growth of pneumococcus in dextrose broth.

100 cc. portions of plain broth containing 1 per cent dextrose were adjusted to the desired pH, and 25 cc. removed for controls. After 24 hours at 37°C. as a sterility test, the 75 cc. were inoculated with 0.5 cc. of a 12 hour broth culture of Pneumococcus Type I–1 (this heavy dose was given in order to complete the experiment within 1 day). At intervals 5 cc. samples were removed for pH determinations, and when these readings indicated active growth 5 cc. samples were autoclaved for nephelometer readings. Macroscopic estimations of growth were also made to a +++ scale. The results are given in Table VII and in Text-fig. 2.

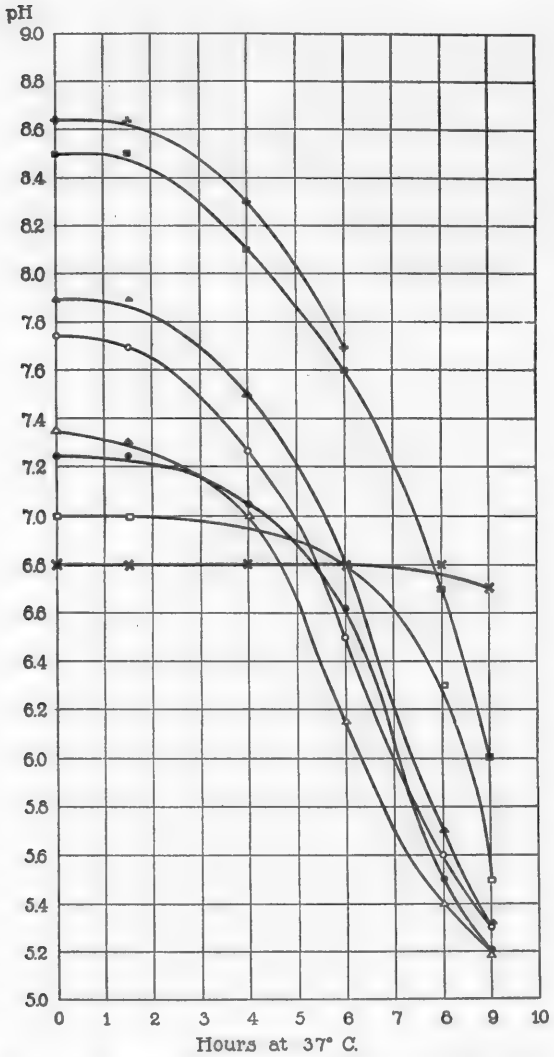
TABLE VII.

Optimum Hydrogen Ion Concentration for Growth of Pneumococcus in Dextrose Broth.

75 cc. of plain broth containing 1 per cent dextrose were inoculated with 0.5 cc. of a 12 hour broth culture of Pneumococcus Type I–1.

Hydrogen ion concentration.						Growth.		Turbidity. 6 hrs.
Control.	1½ hrs.	4 hrs.	6 hrs.	8 hrs.	9 hrs.	1 hr.	6 hrs.	
pH	*pH*	*pH*	*pH*	*pH*	*pH*			*per cent*
6.8	6.8	6.8	6.8	6.8	6.7	−	±	4.2
7.0	7.0	7.0	6.8	6.3	5.5	−	+	32.0
7.25	7.25	7.05	6.6+	5.5	5.2	+	+++	60.0
7.35	7.3	7.0	6.15	5.4	5.2	+	+++	
7.75	7.7	7.3	6.5	5.6	5.3	+	+++	82.0
7.9	7.9	7.5	6.8	5.7	5.3	−	+++	85.0
8.5	8.5	8.1	7.6	6.7	6.0	−	+±−	30.0
8.6+	8.6+	8.3	7.7			−	+	26.0

It is evident from these experiments that the optimum hydrogen ion concentration for growth of pneumococcus in dextrose broth was about pH 7.8; that is, the same as for plain broth (Text-fig. 1). Cultures grow luxuriantly between pH 7.2 and 8.6. The upper limit for growth in sugar medium is somewhat higher than in plain medium. Although fermentation in sugar broth starts at a pH of 7.8 and proceeds until a pH of 5 is reached, it is not possible to initiate growth below pH 7.0. The curve of the rate of acid production for this series has been also constructed (Text-fig. 2). At pH 7.75 to 7.3 the initial lag is decreased, but in all the tests from pH 7.2 to 8.6 after the initial



TEXT-FIG. 2. Effect of initial hydrogen ion concentration upon the rate of reaction change due to growth of pneumococcus in dextrose broth.

lag luxuriant growth occurs at about the same rate. This experiment emphasizes the fact that the term limiting hydrogen ion concentration must be carefully defined and that the final hydrogen ion concentration may be entirely different from the hydrogen ion concentration limits within which growth may be initiated.

Limiting Initial Hydrogen Ion Concentration.—It is brought out in the preceding experiments that the ordinary inoculation of pneumococcus fails to initiate growth if the hydrogen ion concentration of the medium is greater than pH 7.0, but that with growth well started at a pH above 7.0, acid production proceeds in sugar media to a final pH of 5.0. It would seem possible then that either the bacteria rapidly adapt themselves to the changing and hitherto unfavorable reaction, or that the medium itself is rendered more suitable for growth.

If the explanation lies in rapid adaptation of the organism one would expect that bacteria removed from a sugar broth at a reaction of pH 6.0, for example, could grow in fresh broth of the same reaction. An attempt to test this point was carried out as follows:

Dextrose broth was inoculated with 0.5 cc. of an 18 hour culture of Pneumococcus Type I. The change in reaction was followed, and at pH of 7.1, 6.5, 5.5, and 5.0 fresh samples of dextrose broth of varying hydrogen ion concentrations were inoculated with the actively growing culture. This broth had previously been adjusted to the desired reaction, tubed in 5 cc. portions, and incubated to test sterility. The heavy inoculation of 0.1 cc. of culture per 5 cc. of medium was used, and in addition several tubes were inoculated with the massive dose of 0.5 cc. of culture to 5 cc. of medium. The 13 hour culture (pH 5) was still viable as evidenced by the occurrence of growth when inoculated into fresh broth of pH 7.8. Only traces of disintegration of the cells were visible under the microscope. This experiment is recorded in Table VIII.

Study of Table VIII shows that it is impossible to initiate growth in media having a more acid reaction than pH 6.8 regardless of the fact that the inoculum may have been removed from an actively growing culture at ranges of acidity from pH 7.1 to 5.0. For instance, if the organisms are alive and growing at a pH 5.5, and a seeding is removed at this point and implanted in medium with a reaction of pH 6.5, no growth occurs.

TABLE VIII.

Failure of Actively Growing Cultures at pH below 6.8 to Initiate Growth in Fresh Broth of pH below 6.8.

5 cc. of dextrose broth of the indicated reaction were incubated for 24 hours at 37°C. after inoculation with cultures which had developed an acid reaction.

Culture (Type I).		1 per cent dextrose broth.	Inoculation per 5 cc.			
			0.1 cc.		0.5 cc.	
Age.	Hydrogen ion concentration.		Growth.	Final hydrogen ion concentration.	Growth.	Final hydrogen ion concentration.
hrs.	*pH*	*pH*		*pH*		*pH*
6½	7.1	7.8	++++	4.9		
8	6.5		++++	4.8		
9	5.5		++++	4.9		
13	5.0		++++	4.9		
6½	7.1	7.0	−	7.0−		
8	6.5		−	6.8+	++++	4.9
9	5.5		++++	5.0	++++	5.0
13	5.0		++++			
6½	7.1	6.8	−	6.8		
8	6.5		++++	4.9	++++	4.9
9	5.5		−	6.5	++++	4.9
13	5.0		−	6.8		
6½	7.1	6.5	−	6.5		
8	6.5		−	6.3	−	6.1
9	5.5		−	6.35	−	6.1
13	5.0		−	6.4		
6½	7.1	6.0	−	6.0		
8	6.5		−	6.0	−	5.9
9	5.5		−	5.95	−	5.8
13	5.0		−		−	5.8

Growth of Pneumococcus in Filtrates of Dextrose Broth Cultures of Pneumococcus.—An attempt was next made to determine whether dextrose broth in which pneumococcus has grown to the final hydrogen ion concentration will sustain growth if the organisms are removed by Berkefeld filtration and the reaction of the filtrate is readjusted to ranges of acidity from pH 7.8 to 6.0.

To salt-free broth 0.2 per cent sodium phosphate and 1 per cent dextrose were added, the medium was adjusted to a pH 7.8, and inoculated with Type I pneumococcus. After 24 hours incubation the culture was filtered through a Berkefeld filter. Samples of the filtrate, the pH of which was 5.2, were adjusted with sodium hydroxide to pH 6.0, 6.5, 6.8, 7.0, and 8.0; 5 cc. of filtrate required 3.3 cc. of 0.05 N sodium hydroxide to bring it to a pH 8.0. The filtrate at each pH was divided into 5 cc. portions, one of which was used as a test for sterility. Tubes containing 5 cc. of filtrate were inoculated in duplicate with 0.1 cc. of 18 hour cultures of Types I and II. The results are given in Table IX.

TABLE IX.

Growth of Pneumococcus in Filtrates of Dextrose Broth Cultures of Pneumococcus.

Dextrose broth pH 7.8. After 24 hours the culture was filtered through a Berkefeld filter. The pH of the filtrate was 5.2.

Filtrate from Type I (Strain 183) adjusted to.	48 hrs. after inoculation with.			
	Type I (Strain 183).		Type II (Strain D 39).	
	Growth.	Hydrogen ion concentration.	Growth.	Hydrogen ion concentration.
pH		*pH*		*pH*
8.0	++++	5.3		
7.0	++++	5.2	++++	5.1
6.8	++++	5.2	++++	5.2
6.5	−	6.5	−	6.5
6.0	−	6.0	−	6.0

It is evident that filtrates of dextrose broth in which pneumococcus has grown and the reaction of which has been readjusted with sodium hydroxide will not allow growth to be initiated if the readjusted pH is below 6.8.

This experiment indicates that the medium is not specifically exhausted of the substances necessary for growth, nor is there a formation of specific inhibiting bodies. This has been confirmed by growing two other strains of different types of pneumococcus in dextrose broth. After a week the cultures became sterile, the organisms were removed by centrifugation, the supernatant fluids were adjusted to

a pH 7.7, and portions of each fluid were reinoculated from fresh cultures of both organisms. Growth occurred in all portions (Table X).

TABLE X.

Dextrose Broth in Which Pneumococcus Has Grown Shows No Specific Exhaustion of Fermentable Substances and No Specific Inhibiting Substances.

1 per cent dextrose broth, pH 7.7, after 7 days incubation, broth sterile, pH 5.1. The supernatant fluid after centrifugation was adjusted with sodium hydroxide to pH 7.7, reinoculated, and incubated for 48 hours.

Supernatant fluid of broth cultures of pneumococcus.	Adjusted hydrogen ion concentration of supernatant fluid.	Reinoculation of supernatant fluid with pneumococcus.	
		Type I.	Type II.
	pH	*pH*	*pH*
Type I	7.7	5.1	5.1
" II	7.7	5.1	5.1

Exhaustion of Fermentable Substances in Plain Broth.—Since growth cannot be started in either plain broth or in dextrose broth if the hydrogen ion concentration is appreciably greater than pH 7.0 to 6.8, it is evident that this degree of acidity is unfavorable for the initiation of growth. However, pneumococcus in dextrose broth of pH above 7.0 grows to a pH of 5.0, while in plain broth of the same initial pH, growth ceases at about 7.0. Is this cessation of growth a result of the attaining of that reaction, or is it due to exhaustion of fermentable substances? To test this, plain broth cultures of pneumococcus which had developed maximum acidity, were filtered through a Berkefeld filter and the filtrate was divided into several portions and reinoculated with pneumococcus. As seen in Table XI the filtrate, both at pH 7.0 and when readjusted to its initial pH 7.5, shows no growth; on the other hand, the addition of dextrose, whether to the unadjusted filtrate, or to filtrate the reaction of which has been restored to the initial pH, is sufficient to cause abundant growth. It is evident then that the cessation of growth in plain broth when the culture has reached about pH 7.0 is due, partially at least, to exhaustion of fermentable substances. The acid produced in itself does not inhibit growth. This conclusion is emphasized by Table I in which it is shown that 0.1 per cent dextrose broth reaches a pH 6.5, 0.2 per cent dextrose broth a pH 6.0, and 0.4 per cent dextrose broth a pH 5.0.

TABLE XI.

Exhaustion of Fermentable Substances in Plain Broth by Pneumococcus.

100 cc. of plain broth, initial pH 7.5, were inoculated with Type I (Strain D 46), at 37°C. for 24 hours, and filtered through a Berkefeld filter. The filtrate, pH 7.0, was reinoculated and incubated 48 hours.

Filtrate of plain broth culture of Pneumococcus Type I.	Hydrogen ion concentration of filtrate.	Filtrate reinoculated with pneumococcus.			
		Type I.		Type II.	
		Growth.	Hydrogen ion concentration.	Growth.	Hydrogen ion concentration.
	pH		*pH*		*pH*
Unchanged	7.0	−	7.0	−	7.0
Dextrose added to 1 per cent	7.0	+++	5.0	+++	5.1
Adjusted with NaOH	7.5	−	7.5	−	7.5
" " NaOH + 1 per cent dextrose	7.5	+++	5.0	+++	5.0

DISCUSSION.

In this paper are presented facts thus far acquired in a study of acid production by pneumococcus when grown in the presence of fermentable substances. The strains of pneumococcus chosen are representative of the different serological types and in the majority of instances have been isolated from patients having lobar pneumonia. Some of these strains were freshly isolated, others have been under cultivation on artificial media for years. During this time their virulence has been maintained by animal passage and they have lost none of their biologic specificity. It is evident also from the protocols given that these conditions of preservation and animal passage have not affected the biochemical functions concerned in acid production. Moreover, it is apparent, for the limited number of strains studied at least, that no difference in the degree of acidity produced by the specific types of pneumococcus could be determined in media containing the test substances. In measuring the acidity produced by growth of pneumococcus use has been made of the colorimetric method for determining the hydrogen ion concentration. The limit of acid tolerance of pneumococcus in sugar-containing media, representing the final hydrogen ion concentration, is remarkably constant for all the strains studied.

The point of maximum acidity at which growth ceases has been defined as the final hydrogen ion concentration and has been found for all types of pneumococcus in carbohydrate media to be about pH 5. This final reaction is affected by the concentration of fermentable sugar in the medium up to 0.4 per cent. In the presence of this amount of dextrose, for instance, sufficient acid is produced during growth to bring the medium to the final hydrogen ion concentration of pH 5, but excess of sugar up to at least 4 per cent has no further influence on the final reaction. The rate of acid production, while a function of the size of the inoculum and of the optimum reaction of the medium, is, as previously shown by Chesney (9) and Cullen and Chesney (10), after the period of initial lag, rapid and constant until the final hydrogen ion concentration is reached.

In media containing 1 per cent maltose, saccharose, lactose, galactose, raffinose, dextrose, or inulin, strains of pneumococcus representing the four specific types produced acid to a final hydrogen ion concentration of pH 5. These results are in agreement with those of a previous study of 48 strains in which it was observed that these test substances are fermented by pneumococcus regardless of type differences.

Dernby and Avery have previously shown that the optimum hydrogen ion concentration for growth of pneumococcus in plain broth is pH 7.8. In the course of the present experiments these results have been confirmed and the observations extended to determine the optimum reaction for growth in media containing sugar. The initial reaction of all the media tested shows the optimum to be pH 7.8. The limits of hydrogen ion concentration within which growth can be initiated, however, were found to cover a somewhat wider range in carbohydrate-containing media (pH 8.3 to 6.8) than in similar media to which these substances are not added (pH 8.1 to 7.0). However, it is apparently impossible to initiate growth in a medium containing sugar if the initial reaction is more acid than pH 6.8, even though the organisms used for seeding are transplanted immediately from an actively growing culture at ranges of acidity varying from pH 7.1 to 5.

It has been found that in bacteria-free filtrates of plain broth cultures of pneumococcus, even when the filtrate is adjusted to the opti-

mum reaction, pneumococcus cannot be made to grow again unless small amounts of sugar (dextrose) are added. On the other hand, filtrates of dextrose broth cultures of pneumococcus, under the optimum conditions of reaction, apparently contained sufficient unutilized sugar to allow growth to occur on subsequent reinoculation. The ability of pneumococcus to grow, then, when reinoculated into the filtrate of a broth culture of pneumococcus of the same or different type, after the reaction of the filtrate has been readjusted to the optimum hydrogen ion concentration, appears to be dependent in part at least upon the presence of a residuum of fermentable substance left unmetabolized by previous growth. It is probable, however, that the exhaustion of fermentable carbohydrate from culture media is only one of many factors involved in the complex phenomenon of growth inhibition.

SUMMARY.

1. The optimum hydrogen ion concentration for growth of pneumococcus is pH 7.8.

2. In broth cultures growth of pneumococcus continues until a final hydrogen ion concentration of about pH 5.0 is reached, if sufficient fermentable carbohydrate (above 0.4 per cent) is present. Apparently this acidity is sufficient in itself to stop growth.

3. If less carbohydrate is present in the medium growth ceases at a lower hydrogen ion concentration, apparently because of exhaustion of carbohydrate. If no carbohydrate is present save that extracted from the meat of which the broth is made (plain broth medium), growth initiated at pH 7.8 (optimum reaction) ceases at about pH 7.0.

4. If bacteria-free filtrates of plain broth cultures in which growth has ceased are readjusted to pH 7.8 and reinoculated with pneumococcus, no growth occurs unless carbohydrate is added. However, if bacteria-free filtrates of dextrose broth cultures in which growth has ceased (pH 5) are readjusted to pH 7.8 and reinoculated with pneumococcus growth occurs.

5. Cultures of pneumococcus with all the carbohydrates which were fermentable under the conditions used, namely maltose, saccharose, lactose, galactose, raffinose, dextrose, and inulin, gave identical results in the rate of reaction change, and final hydrogen ion concentration (pH 5.0) attained.

6. The different immunological types of pneumococcus, for the limited number of strains studied, behaved alike in fermenting the carbohydrates mentioned above.

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THE RELATION OF PROTEOLYTIC ENZYMES IN THE PNEUMONIC LUNG TO HYDROGEN ION CONCENTRATION. AN EXPLANATION OF RESOLUTION.

By FREDERICK T. LORD, M.D.

(*From the Research Laboratory of the Massachusetts General Hospital, Boston.*)

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The relation of the pneumococcus to the production of acid in culture media and the acid reaction of the pneumonic lung[1] suggest that crisis and recovery may be due to local biochemical changes in the course of which the acid death-point of the pneumococcus is reached. In this paper evidence is presented of certain factors influencing resolution in pneumonia. Dernby's work on the autolysis of animal tissue[2] suggested methods applicable to the investigation.

Three fatal cases (Cases 1, 2, and 3) of pneumococcus pneumonia in the stage of gray or gray-red hepatization furnished the material. The involved lung was passed through a nut butter cutter, the resulting fluid from the mash filtered through cheese-cloth, and the filtrate centrifuged. The grayish sediment was washed in normal saline solution and recentrifuged. The material thus obtained is spoken of below as cellular material. Microscopic examination showed numerous pus cells, many large mononuclear cells, and numerous pneumococci. Chloroform was added to this material to kill the bacteria. Cultures made after the addition of chloroform were sterile. For all the experiments the cellular material was diluted. The diluted mixture is called cellular suspension. This cellular suspension used as enzyme proved much more active than the clear fluid which separates on standing, suggesting that proteolytic action is due to a ferment liberated by the cells.

A special object of the investigation was to determine the influence

[1] Lord, F. T., *Tr. Am. Soc. Clin. Investigation*, 1916, 8; *J. Am. Med. Assn.*, 1916, lxvii, 1981; 1919, lxxii, 1364.

[2] Dernby, K. G., *J. Biol. Chem.*, 1918, xxxv, 179.

of varying hydrogen ion concentration on the proteolytic activity of the cellular material.

Erosion of Blood Serum.—A proteolytic enzyme eroding the surface of Löffler's blood serum was readily demonstrated in the cellular material obtained from the pneumonic exudate in all three cases. The addition of chloroform to the cellular material excluded the action of living bacteria. The erosion of the surface of blood serum by the action of cellular material from various sources including the pneumonic exudate is a frequent observation in the laboratory. The relation of the proteolysis to varying degrees of acidity of the medium is an important matter.

Experiment 1.—Tubes of slanted blood serum at varying hydrogen ion concentrations were made as indicated in Table I.

TABLE I.

Method of Preparing Slanted Löffler's Blood Serum at Varying Hydrogen Ion Concentrations for Testing Proteolytic Action.

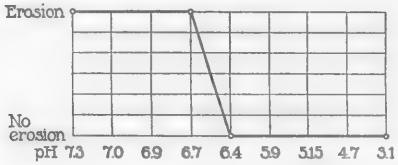
Blood serum 750 cc., dextrose bouillon 250 cc., glycerol 15 cc. To 30 cc. of this mixture was added the indicated amount of N hydrochloric acid or 0.1 N sodium hydroxide + water to 36 cc.

Flask No.	N HCl	0.1 N NaOH	Water.	pH
	cc.	cc.	cc.	
a	Slanted blood serum immersed in solutions of known hydrogen ion concentration.*			3.1
b				4.7
1	2.0		4.0	5.15
2	0.9		5.1	5.9
3	0.7		5.3	6.4
4	0.5		5.5	6.7
5		2.0	4.0	7.0
6		4.5	1.5	7.3

* The creation of a higher acidity than 5.1 by the addition of acid to the serum prevented the inspissation of the medium and slants of coagulated blood serum already made were allowed to stand covered with standard solutions containing 0.2 M acid potassium phthalate or 0.2 M potassium dihydrogen phosphate and 0.2 M sodium hydroxide of hydrogen ion concentration varying from 4.6 to 2.2. Samples of the fluid were removed at intervals until the hydrogen ion concentration had become constant. By this means, after 3 days slants were obtained of Löffler's blood serum, the fluid covering which had a pH of 3.1 to 5.15.

The water of condensation was removed before the experiment. Cellular material obtained from Case 3 was used. The cellular suspension was made by suspending four drops of cellular material in ten drops of standard solutions of known hydrogen ion concentration and the hydrogen ion concentration of the resulting cellular suspension was then determined. To the surface of the slanted blood serum[3] of varying hydrogen ion concentrations a cellular suspension of nearly the same hydrogen ion concentration was added. After incubation erosion of the surface of the medium was observed on the slants with a pH of 7.3 to 6.7 (Text-fig. 1).

Other experiments confirmed these results and showed that the precaution of controlling the hydrogen ion concentration of the cellular suspension used as enzyme was unnecessary. The enzyme proved active at and below 6.7 and inactive at and above 6.4; *i.e.*,



TEXT-FIG. 1. Proteolytic enzyme eroding the surface of Löffler's blood serum. To slanted blood serum with water of condensation of hydrogen ion concentrations varying from 7.3 to 3.1 cellular material suspended in solutions of approximately the same hydrogen ion concentration was added. Erosion of the medium was noted in the slants with a pH of 7.3 to 6.7. No erosion occurred at a pH of 6.4 or higher.

on the more acid end of the scale. Chloroform was added to the cellular material and no growth of bacteria was observed on the surface of the medium.

Gelatin Method.—A stock solution was made by dissolving 350 gm. of gelatin in 625 cc. of hot water and the solution passed through cheese-cloth. 1 gm. of thymol suspended in water was added and the solution made up to 1 liter with water.

Before the experiments 200 cc. of this stock solution were taken and diluted to 500 cc. To 15 cc. of this solution hydrochloric acid or sodium hydroxide was added to vary the hydrogen ion concentrations and the volume was made up to 30 cc. with distilled water. Two series of gelatin solutions with hydrogen ion concentrations

[3] Kept horizontal after the addition of the cellular suspension.

varying from 8.0 to 2.0 were thus prepared, one to serve as control and the other for the experiment.

Experiment 2.—To each flask in one of the two series three drops of the cellular suspension obtained from the pneumonic lung (Case 1) and used as enzyme were added. The cellular suspension was made from 0.5 cc. of cellular material (to which chloroform had been added) diluted with 10 cc. of sterile distilled water. Cultures from the cellular material showed no growth. Both control and experimental solutions were placed in the incubator. After 4½ and 22 hours 5 cc. samples were removed from each of the flasks in the two series, placed in thin test-tubes, and cooled for 15 minutes in ice water. On removal from the ice bath the degree of liquefaction of the gelatin in the two series was compared. For the purpose of recording the result the tubes found completely solid were classed as 0 and those completely liquid as 6, intervening numbers indicating a corresponding degree of liquefaction. Cultures from the experimental solutions at the termination of the experiment showed no growth.

Experiment 3.—An exactly similar experiment was performed with cellular material obtained from the pneumonic lung in Case 2.

The presence of an enzyme acting at both acid and alkaline ends of the scale was suggested by some degree of liquefaction of the gelatin in the experimental flasks, but the curve showed a tendency to rise progressively toward the ends of the scale, making the interpretation doubtful. The experiments with gelatin lose in value on account of an almost exactly similar behavior of the controls. Loeb[4] has shown that gelatin at its isoelectric point ($C_H = 2.10^{-5}$) is practically insoluble, but when it is transformed into a salt by the addition of an acid (or a base) it becomes soluble provided it is in combination with a monovalent ion. Although the gelatin can be brought back to the critical hydrogen ion concentration by adding a calculated amount of acid or alkali before the samples are placed in the ice bath, this procedure further complicates the experiment and does not seem to offer more advantage than the use of the controls. The presence of an enzyme liquefying gelatin could not be demonstrated by this method.

Peptone Method.—Seven solutions of Witte's peptone with hydrogen ion concentrations varying from a pH of about 8.5 to 4.0 were prepared according to the method indicated in Table II. To por-

[4] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 363.

tions removed from each of the seven flasks equal amounts of a homogeneous cellular suspension used as enzyme were added. The amino-acid nitrogen in the solutions was determined by Van Slyke's method[5] with the micro-apparatus devised by him.[6] The cells were first removed by centrifuging the solutions. The total nitrogen determinations were done in duplicate by Folin and Denis' method,[7] by direct Nesslerization, or by the Kjeldahl method.

TABLE II.

Method of Preparing Peptone at Varying Hydrogen Ion Concentrations for Testing Proteolytic Action.

20 cc. of 4 per cent peptone (Witte's) + the indicated amount of 0.5 M phosphates + hydrochloric acid or sodium hydroxide + distilled water to 90 cc.

Flask No.	Chloroform.	0.1 N HCl	0.1 N NaOH	0.5 M phosphates.		pH indicated.	pH determined.
				KH_2PO_4	Na_2HPO_4		
	cc.	*cc.*	*cc.*	*cc.*	*cc.*		
1	1	16.0		10.0	0	4.0	4.0
2	1	8.0		9.0	1.0	5.2	5.2
3	1	3.0		5.0	5.0	6.8	6.6
4	1			2.0	8.0	7.3	7.3
5	1		3.0	1.0	9.0	7.7	7.8
6	1		8.0	0.5	9.5	8.0	8.0
7	1		14.0	0	10.0	8.5	8.5

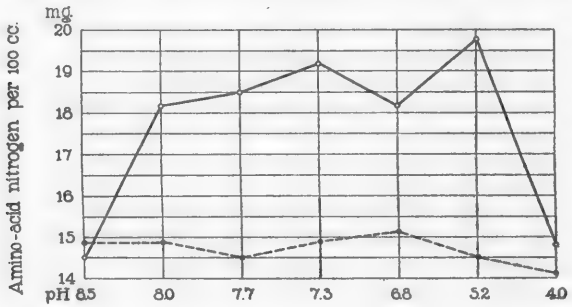
The composition of the solutions used for the determination of peptone-splitting enzyme in the pneumonic lung was the same as that used by Dernby in his study of autolysis of animal tissue with the exception of a change in the amount of peptone from 40 cc. to 20 cc. and the water from 180 cc. to 90 cc.

Experiment 4.—To 0.5 cc. of cellular material obtained from a Pneumococcus Type II pneumonia (Case 1) chloroform was added and the material was allowed to stand over night to plasmolyze. Cultures made after the addition of the chloroform were sterile. A cellular suspension was made by adding to the 0.5 cc. 16 cc. of distilled water. 28 cc. from each of the seven flasks (Table II) were removed to separate flasks and to each were added 2 cc. of the homogeneous cellular suspension used as enzyme and four drops of chloroform. The remainder

[5] Van Slyke, D. D., *J. Biol. Chem.*, 1912, xii, 275.
[6] Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxiii, 407.
[7] Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 473.

of the original peptone solutions to serve as controls and the experimental solutions were allowed to remain in the incubator for 44 hours. The results of the determination of the amino-acid nitrogen are indicated in Table III and graphically recorded in Text-fig. 2.

As indicated in Table III the control solutions already contained from 14.3 to 15.1 mg. of amino-acid nitrogen per 100 cc., the slight variations in the different flasks indicating the experimental error which does not exceed 0.8 mg. per 100 cc. Though equal amounts of homogeneous cellular suspension were added to the experimental flasks the resulting increase in the amino-acid nitrogen varies at the different hydrogen ion concentrations and reaches a maximum in the



TEXT-FIG. 2. Peptone-splitting enzyme in the pneumonic lung from Case 1. The action of cellular material obtained from pneumonic lung in splitting Witte's peptone to amino-acid nitrogen after incubation for 44 hours is shown. The solid line indicates solutions with cellular material, the broken line the same solutions without cellular material (control).

flask with a hydrogen ion concentration of 5.2 pH, falling sharply from this point toward the more acid side and slowly toward the more alkaline side until a pH of 8.0 is reached when the curve again falls abruptly. The lack of variation beyond the experimental error in the control flasks under the conditions of the experiment and the much wider variations at the different hydrogen ion concentrations in the experimental flasks suggest that an enzyme of optimum activity at a pH of 5.2 is the cause of the increased liberation of amino-acid nitrogen. The experiment shows that the amount of amino-acid nitrogen in the peptone solutions without cellular suspension does not

TABLE III.

Peptone-Splitting Enzyme in the Pneumonic Lung from Case 1.

Flask No.	Initial pH.	Control. Amino-acid nitrogen per 100 cc.	Experiment. Amino-acid nitrogen per 100 cc.	Cultures from flasks.	
				Before experiment.	At end of experiment.
		mg.	*mg.*		
1	4.0	14.3	14.7	No growth.	No growth.
2	5.2	14.5	19.9	" "	" "
3	6.8	15.1	18.2	" "	5 white colonies.
4	7.3	14.7	19.1	" "	No growth.
5	7.7	14.5	18.5	" "	" "
6	8.0	14.7	18.2	" "	" "
7	8.5	14.7	14.5	" "	" "

Control solution, total nitrogen (Folin and Denis method) 90 mg. per 100 cc. The five colonies obtained from the transplant from No. 3 were a contaminating organism, not pneumococci.

appreciably change. The experiment is open to the criticism that amino-acid nitrogen preformed in the cellular suspension may have been added to the peptone solutions, but other experiments have shown that there is too small an amount of preformed amino-acid nitrogen in the $\frac{1}{16}$ cc. of cellular material added to each flask to influence the individual readings. Any possible error from this source is eliminated in the following experiments.

Experiment 5.—An experiment was performed using cellular material obtained from another fatal case (Case 3) of Pneumococcus Type I pneumonia. The cellular material treated as before with chloroform was sterile. The cellular suspension was made by diluting 0.5 cc. of cellular material with 16 cc. of distilled water. 2 cc. of the cellular suspension were added to 28 cc. from each of seven freshly prepared solutions of Witte's peptone of the composition indicated in Table II. 0.3 cc. of chloroform was added to each flask.

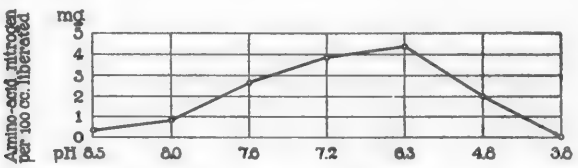
The amount of amino-acid nitrogen in each flask averaged 14.3 mg. per 100 cc. before the addition of the cellular material. The total nitrogen (Kjeldahl method) in the peptone solutions averaged 103.5 mg. per 100 cc. before and 106 mg. after the addition of the cellular suspension. The cellular suspension therefore added about 2.5 mg. of total nitrogen per 100 cc. This amount of total nitrogen in the cellular suspension is too small to account for more than a fraction of the amino-acid nitrogen liberated.

The amount of amino-acid nitrogen in each flask was determined before and after incubation for 42 hours. The results are indicated in Table IV. The increase in amino-acid nitrogen liberated is graphically shown in Text-fig. 3. The hydrogen ion concentration of the flasks changes very little during the experiment. Cultures from the flasks during the experiment were sterile. A peptone-splitting enzyme acting most strongly at a hydrogen ion concentration of 6.3 is indicated.

TABLE IV.

Peptone-Splitting Enzyme in the Pneumonic Lung from Case 3.

Flask No.	Chloroform.	Initial pH.	Final pH.	Amino-acid nitrogen per 100 cc.		Cultures during experiment.
				Before incubation.	After incubation.	
	cc.			*mg.*	*mg.*	
1	0.3	4.0	3.8	14.9	14.9	No growth.
2	0.3	4.9	4.8	15.6	17.6	" "
3	0.3	6.5	6.3	15.0	19.4	" "
4	0.3	7.1	7.2	14.6	18.5	" "
5	0.3	7.6	7.6	15.5	18.4	" "
6	0.3	8.0	8.0	15.2	16.5	" "
7	0.3	8.5	8.5	14.5	15.1	" "



TEXT-FIG. 3. Peptone-splitting enzyme in the pneumonic lung from Case 3. The increase in amino-acid nitrogen at varying hydrogen ion concentrations in flasks containing Witte's peptone and cellular material from the pneumonic lung after incubation for 42 hours is shown.

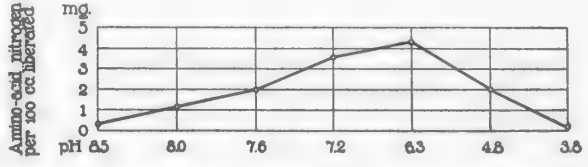
Experiment 6.—0.5 cc. of cellular material similarly obtained from another fatal case of Pneumococcus Type I pneumonia (Case 2) and treated as before with chloroform was diluted with 16 cc. of distilled water. Cultures from the cellular material after the addition of chloroform were sterile. 2 cc. of the cellular suspension were added to 28 cc. from each of the seven original solutions of Witte's peptone used in Experiment 5. The amino-acid nitrogen in the peptone solutions was already 14.3 mg. per 100 cc.; the total nitrogen (Kjeldahl method) averaged 103.5 mg. per 100 cc. before and 105 mg. per 100 cc. after the addition of the cel-

lular suspension, indicating that the cellular suspension added about 1.5 mg. of total nitrogen per 100 cc. This amount of total nitrogen in the cellular suspension is too small to account for more than a small fraction of the amino-acid nitrogen liberated from the Witte's peptone. The amount of amino-acid nitrogen was determined in each flask before and after incubation for 42 hours, with the result recorded in Table V and graphically presented in Text-fig. 4. As in the preceding experiment a peptone-splitting enzyme acting most strongly at a hydrogen ion concentration of 6.3 is indicated. Cultures from the flasks before, during, and after the experiment were sterile.

TABLE V.

Peptone-Splitting Enzyme in the Pneumonic Lung from Case 2.

Flask No.	Chloroform.	Initial pH.	Amino-acid nitrogen per 100 cc.		Cultures before, during, and after experiment.
			Before incubation.	After incubation	
	cc.		*mg.*	*mg.*	
1	0.3	3.8	14.8	15.0	No growth.
2	0.3	4.8	14.7	16.7	" "
3	0.3	6.3	14.5	18.8	" "
4	0.3	7.2	14.8	18.2	" "
5	0.3	7.6	15.0	17.0	" "
6	0.3	8.0	15.0	16.1	" "
7	0.3	8.5	14.5	14.8	" "



TEXT-FIG. 4. Peptone-splitting enzyme in the pneumonic lung from Case 2. The increase in amino-acid nitrogen at varying hydrogen ion concentrations in flasks containing Witte's peptone and cellular material from the pneumonic lung after incubation for 42 hours is shown.

SUMMARY.

Evidence is given of the presence in the cellular material obtained from the pneumonic lung of a proteolytic enzyme digesting coagulated blood serum at hydrogen ion concentrations of 7.3 to 6.7 and inactive at higher; *i.e.*, more acid concentrations.

In addition, evidence is brought forward of the presence in the cellular material from the pneumonic lung of a proteolytic enzyme splitting peptone to amino-acid nitrogen. This enzyme is operative at hydrogen ion concentrations from 8.0 to 4.8, but most active at 6.3 or 5.2.

These findings may be regarded as having a bearing on resolution in pneumonia. During the course of the disease a gradual increase in the hydrogen ion concentration of the exudate probably takes place. With the breaking down of cellular material an enzyme digesting protein (fibrin) in weakly alkaline and weakly acid media may be liberated. With a gradual increase in the hydrogen ion concentration of the pneumonic lung the action of this enzyme probably ceases. An enzyme capable of splitting peptone to amino-acid nitrogen is probably active during the proteolysis of the fibrin and further activated when the hydrogen ion concentration of the pneumonic lung is increased to within its range of optimum activity at a pH of 6.3 and 5.2. By this means it may be conceived that the exudate is dissolved and resolution takes place.

I am indebted to Dr. R. N. Nye for assistance, to Miss A. S. Minot for the chemical analyses, and to Miss E. W. Bicknell and Miss Margaret Herrick for technical aid.

THE RELATION OF THE PNEUMOCOCCUS TO HYDROGEN ION CONCENTRATION, ACID DEATH-POINT, AND DISSOLUTION OF THE ORGANISM.*

By FREDERICK T. LORD, M.D., and ROBERT N. NYE, M.D.

(From the Research Laboratory of the Massachusetts General Hospital, Boston.)

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Lord[1] has called attention to the probable importance of an increase in the hydrogen ion concentration in the pneumonic lung in inhibiting the growth of the pneumococcus and favoring enzymatic action. In this article we wish to elaborate certain aspects of the relation of the pneumococcus to changes in acidity. The hydrogen ion concentrations were determined by the colorimetric method with standard solutions made according to Clark and Lubs' directions.[2] As indicators, phenolsulfonephthalein was used on hydrogen ion concentrations ranging from 8.0 to 6.2, and sodium alizarin sulfonate from 6.0 to 4.0. Colored solutions were either dialyzed or the determinations made by the comparator rack method.

Relation of the Pneumococcus to Varying Hydrogen Ion Concentrations.

Experiment 1.—A 1 per cent glucose bouillon culture of Pneumococcus Type II with an initial hydrogen ion concentration of 7.65 shows an increasing multiplication of the organism, as indicated in Text-fig. 1, and reaches a maximum of growth in about 12 hours. The acidity increases to a pH of 5.25 at which there is a rapid fall in the number of living pneumococci. With the increase in the acidity to a pH of 5.15 no living organisms remain in the flask.

Reinoculation of such a flask in which the pneumococcus has grown and died out and the removal of samples at intervals show that living

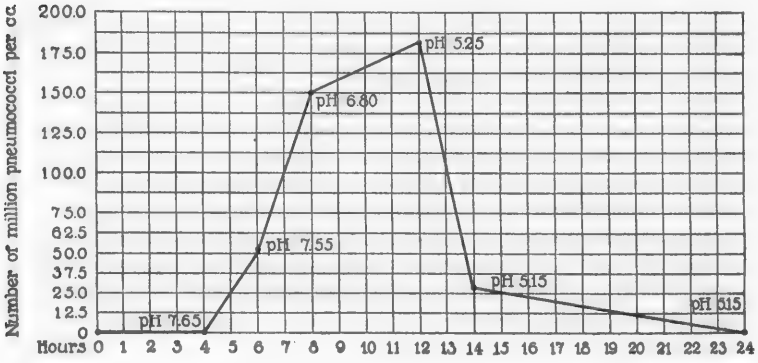
* Presented at the meeting of the American Society for Clinical Investigation, Atlantic City, N. J., June 14, 1919.

[1] Lord, F. T., *Tr. Am. Soc. Clin. Investigation*, 1916, 8; *J. Am. Med. Assn.*, 1916, lxvii, 1981; 1919, lxxii, 1364.

[2] Clark, W. M., and Lubs, H. A., *J. Biol. Chem.*, 1916, xxv, 479.

organisms can be obtained from the flask for an interval of 1 hour and that no growth is obtained on the transplants taken on and after 3 hours. This experiment suggests that a hydrogen ion concentration of about 5.15 may be regarded as the degree of acidity which will almost immediately kill the organism, but the suggestion is open to the objection that other factors than the acidity may be present and exert a bactericidal action.

Other strains of pneumococci of Types I, II, and III, allowed to grow in 1 per cent glucose bouillon until the culture becomes sterile,



TEXT-FIG. 1. The acid death-point of Pneumococci Type II. The results with 1 per cent glucose bouillon are shown.

produce a final acidity of about the same hydrogen ion concentration. No noteworthy difference among the three fixed types has been noted, with the following exception. One strain of Type I pneumococcus obtained from a fatal case of pneumonia remained alive for at least 10 days after the culture had reached a hydrogen ion concentration of 4.5.

Experiment 2.—Repeated reinoculation of the flask (Experiment 1) after the addition of a sufficient amount of sodium hydroxide to neutralize the titrable acidity results in growth for progressively longer intervals as shown in Table I. Partial exhaustion of the material from which the acid is formed may be regarded as the explanation of the increased viability of the culture.

The experiment suggests that the acidity is the most important factor in the death of the organism since without other change in the conditions of the experiment than the neutralization of the acidity and the lapse of time, the pneumococcus will repeatedly grow in the same flask and for progressively longer periods. However, some other factor than the acidity may also play a part in the death of the organism.

TABLE I.

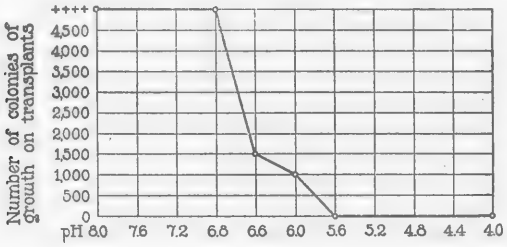
Growth of Pneumococcus in Realkalinized Glucose Bouillon.

Inoculation.	pH	N NaOH required to neutralize 5 cc.	Duration of growth.
		cc.	
1st	7.65–5.15	0.22	14–24 hrs.
2nd	–5.9	0.17	4 days.
3rd	–6.9	0.115	6 "
4th	–7.0		22+ "

1 per cent glucose boullion inoculated with Type II pneumococcus allowed to die out and repeatedly realkalinized and reinoculated.

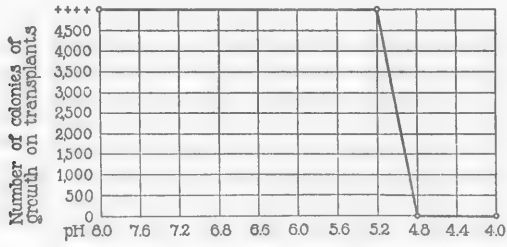
Experiment 3.—In order to test the relation of the pneumococcus to the acidity of the media and to exclude as far as possible the presence of inhibiting substances arising as a result of the growth of the organism, suspensions of washed living pneumococci were made in standard solutions of known hydrogen ion concentrations as follows: Ten drops of each of Clark and Lubs' solutions were placed in small tubes and sterilized in the Arnold sterilizer. Ten drops of a suspension in normal saline solution of the bacterial sediment washed with normal saline solution from an actively growing culture of the pneumococcus were added to each of the solutions of varying hydrogen ion concentrations. The culture of the pneumococcus (Type II) was at the height of its growth and there was no evidence of agglutination or sedimentation in the flask. The addition of the bacteria made a slightly cloudy suspension. The hydrogen ion concentration was not changed by the procedure. The tubes were placed in the incubator and transplants made to the surface of blood serum after 5½ hours, by smearing one loop of the material on the surface, with the result indicated in Text-fig. 2. No growth of organisms was obtained from the tubes at 5.6 or higher hydrogen ion concentrations. An abundant growth of colonies, too numerous to count, was obtained from the tubes at 6.8 and lower hydrogen ion concentrations. About 1,000 colonies were obtained from the tube with a pH of 6.0 and 1,500 from that with a pH of 6.6.

The experiment suggests that irrespective of any bactericidal substances which may be formed in culture media in consequence of the bacterial growth, the pneumococcus will not live for 5½ hours in the presence of hydrogen ion concentrations from 5.6 to 4.0 under the conditions of the experiment, and that some inhibition is present in concentrations from 5.6 to 6.8, beyond which, toward the alkaline end of the scale, more living organisms are present.



TEXT-FIG. 2. The acid death-point of Pneumococci Type II. Suspensions were made in normal saline solution of washed pneumococci at different hydrogen ion concentrations. Transplants were made from these solutions after 5½ hours in the incubator.

Experiment 4.—In Text-fig. 3 the result of a similar experiment with Type III pneumococcus is graphically presented. Bactericidal action of hydrogen ion concentrations from 4.8 to 4.0 acting for 1 hour is indicated.



TEXT-FIG. 3. The acid death-point of Pneumococci Type III. Suspensions were made in normal saline solution of washed pneumococci at different hydrogen ion concentrations. Transplants were made from these solutions after 1 hour in the incubator.

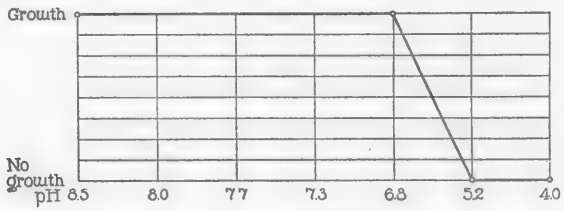
Experiment 5.—To avoid the possibility that the particular solutions used in these experiments (Nos. 3 and 4) may have influenced the result, the experiment was repeated using peptone solutions,[3] of the composition indicated in Table II, of hydrogen ion concentrations varying from 8.5 to 4.0. A large amount of suspension of washed pneumococci (Type II) was added to small amounts of these solutions. After 19 hours in the incubator growth was obtained in transplants from the solutions with a pH of 8.5 to 6.8 as shown in Text-fig. 4. No growth was

TABLE II.

Composition of Solutions for the Determination of the Acid Death-Point.

20 cc. of 4 per cent peptone (Witte's) + hydrochloric acid or sodium hydroxide + 0.5 M phosphates + water to 90 cc.

Flask No.	0.1 N HCl	0.1 N NaOH	0.5 M phosphates.		pH
			KH_2PO_4	Na_2HPO_4	
	cc.	*cc.*	*cc.*	*cc.*	
1	16.0		10.0		4.0
2	8.0		9.0	1.0	5.2
3	3.0		5.0	5.0	6.8
4			2.0	8.0	7.3
5		3.0	1.0	9.0	7.7
6		8.0	0.5	9.5	8.0
7		14.0		10.0	8.5



TEXT-FIG. 4. The acid death-point of Pneumococci Type II. 7.5 cc. of suspensions of washed pneumococci in normal saline solution were added to 2.5 cc. of peptone solutions at hydrogen ion concentrations from 8.5 to 4.0 obtained by adding varying amounts of 0.1 N hydrochloric acid or 0.1 N sodium hydroxide and 0.5 M phosphates. Incubation was for 19 hours. Growth was obtained on transplants from solutions with pH 8.5 to 6.8 and no growth with pH 5.2 to 4.0.

[3] The composition was suggested by Dernby's work (Dernby, K. G., *J. Biol. Chem.*, 1918, xxxv, 179).

obtained from those at 5.2 and 4.0. The experiment indicates that in solutions at a pH of 6.8 and a lower hydrogen ion concentration Pneumococcus Type II will live for 19 hours.

While it is apparent from these experiments that the acidity of the medium has an important bearing on the death of the pneumococcus, it is desirable to obtain data on the relation of the time of exposure to the death of the organism.

Experiment 6.—For this purpose weak solutions of bouillon at varying hydrogen ion concentrations were prepared according to the method indicated in Table III. 5 cc. from each flask were removed to sterile test-tubes. The tubes were

TABLE III.

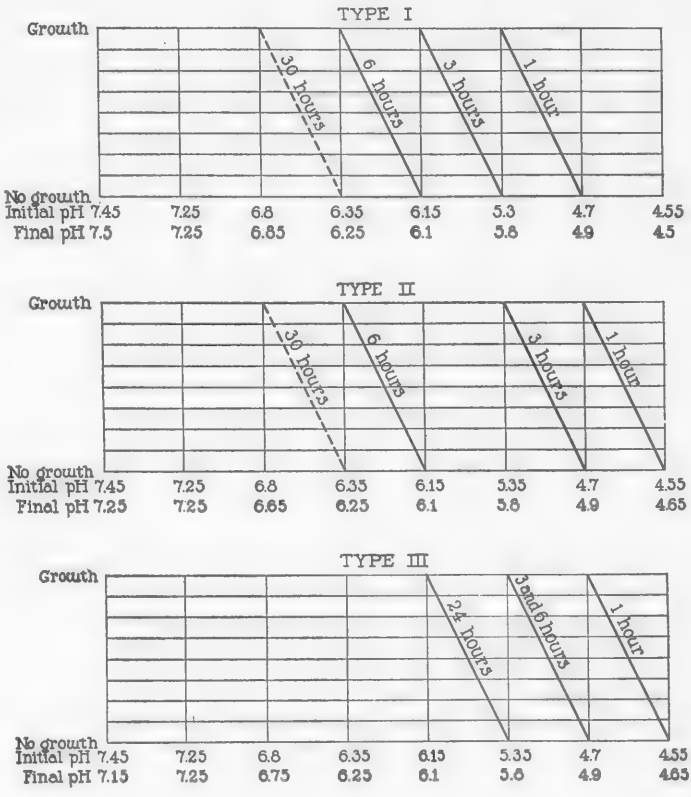
Composition of Solutions for the Determination of the Acid Death-Point.

20 cc. of plain bouillon, hydrochloric acid, and 0.5 M phosphates + water to 90 cc.

Flask No.	0.1 N HCl	0.5 M phosphates.		pH
		KH_2PO_4	Na_2HPO_4	
	cc.	*cc.*	*cc.*	
1	12.0	10.0	0.0	4.55
2	10.0	9.5	0.5	4.7
3	8.0	9.0	1.0	5.35
4	6.0	7.5	2.5	6.15
5	4.0	6.5	3.5	6.35
6	2.0	4.0	6.0	6.8
7	0	2.0	8.0	7.25
8	0	0.0	0.0	7.45

set up in triplicate and one drop of a suspension in salt solution of washed pneumococci of Type I, II, or III was added to each tube of the three series. The tubes were then placed in the incubator and transplants made on blood serum at intervals of 1, 3, and 6 hours. It is apparent from the graphic presentation of the results in Text-fig. 5 that there is a time element in the bactericidal action of acidity. Thus Type I was killed by exposure to a hydrogen ion concentration of 4.5 and 4.7 for 1 hour, but survived an acidity of 5.3 for this interval only to succumb to it after exposure for 3 hours. A pH of 6.15 was withstood for 3 but not for 6 hours and living organisms were obtained after this interval at a pH of 6.35. Type II showed slightly less susceptibility to acid in this experiment. Type III is still less susceptible. Other experiments indicate that there is no constant difference in the acid death-point of the fixed types. Determination of the hydrogen ion concentrations of the mixtures before the experiment and 24 hours later showed no noteworthy change.

From these and other experiments it may in general be said that in fresh culture media the pneumococcus withstands a pH of about 5.3 for 1 hour, of 5.6 for 3 hours, and of 6.1 for 6 hours. It is to be noted (Experiment 1) that when reinoculated into culture media in which the organism has grown and died out it survives a pH of about 5.1



TEXT-FIG. 5. The relation of time and acidity to the death of pneumococci. Suspensions of pneumococci in normal saline solution were added to bouillon solutions at hydrogen ion concentrations varying from 7.45 to 4.5. Transplants were made after varying intervals. The 30 hour periods in Types I and II are taken from another similar experiment.

for 1 hour, thus showing by comparison that there is no essential difference in the acid death-point in fresh culture media and that in culture media containing whatever products may be liberated by the growth and death of the organisms. The acidity is therefore the most important factor.

In contrast to the preceding experiments the following experiments indicate that cultures of pneumococci remain viable when the acidity is low.

Experiment 7.—1 per cent glucose calcium carbonate bouillon with an initial pH of 7.4 was inoculated with pneumococci June 10, 1915. Transplants every few days to June 28 showed growth. On June 28 the hydrogen ion concentration was 6.7. Transplants at less frequent intervals showed growth through December 15. The hydrogen ion concentration was not again determined. The total duration of life was about 6 months.

Experiment 8.—Plain bouillon with an initial pH of 7.4 was inoculated with pneumococci June 10, 1915. Transplants every few days to June 28 showed growth. The pH was then 7.4 as before. Further transplants gave growth through December 15. The hydrogen ion concentration was not again determined. The total duration of life was about 6 months.

Experiments 6 to 8 suggest that within the range of hydrogen ion concentrations investigated (pH 7.45 to 4.5) the pneumococcus is killed by hydrogen ion concentrations above about 6.8 with a rapidity which bears a direct relation to the hydrogen ion concentration, *i.e.* the greater the hydrogen ion concentration the more rapid the death, and that the pneumococcus will continue to live in suitable culture media at a pH of about 6.8 to 7.4 for at least many days.

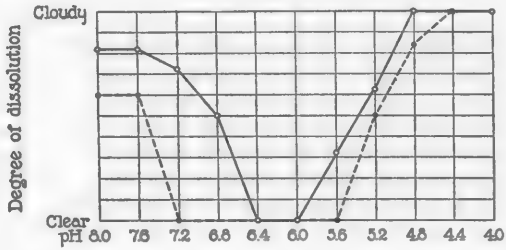
The following experiments suggest that some other factor than the acidity alone is responsible for destruction of pneumococci.

Influence of Varying Hydrogen Ion Concentrations on Dissolution of Pneumococci.

A striking phenomenon which may be spoken of as dissolution is observed when to ten drops of standard solutions of known hydrogen ion concentration ten drops of a suspension of washed pneumococci are added and the resulting slightly cloudy suspension of pneumococci is placed in the incubator. A clearing of the tubes at a hydrogen ion concentration of about 5.0 to 6.0 is observed. The tubes on the more

acid side of the scale remain homogeneously cloudy, although there may be some diminished density at 4.4 and 4.8 and there is constantly some clearing which increases with time on the more alkaline side of the scale. The clearing of the suspension at different hydrogen ion concentrations is graphically indicated in Text-figs. 6 to 8.

Microscopic examination of films from the different tubes shows that the pneumococci in the tubes at the acid end of the scale maintain their morphology, but for the most part become Gram-negative. Examination of films from the cleared suspensions within the range of a pH of about 5.0 to 6.0 shows a few poorly stained organisms, with the shadowy remains of others. The pneumococci are disintegrated and many remnants show as staining points. All are decolorized by

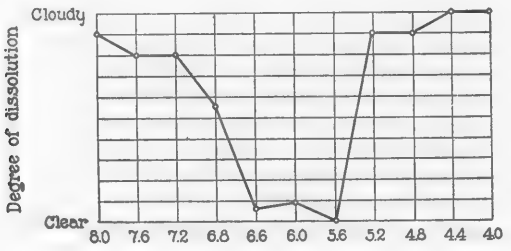


TEXT-FIG. 6. The degree of dissolution of Pneumococci Type I after 7 hours (solid line) and 24 hours (broken line). The suspensions of washed pneumococci in normal saline solution were added to solutions of different hydrogen ion concentrations.

Gram's stain. Films from the tubes at the alkaline end of the scale show some but much less disintegration. Many organisms retain the Gram stain.

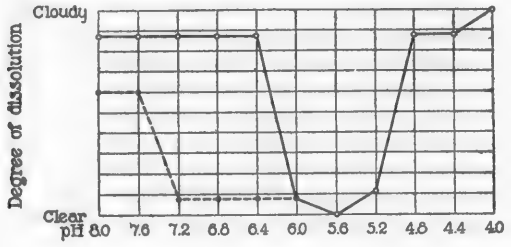
The explanation of the dissolution of the organisms is uncertain. It does not seem to be due to the particular chemical substances, sodium chloride, acid potassium phthalate, potassium dihydrogen phosphate, and sodium hydroxide used in this experiment, since the sodium hydroxide is the only variable factor and the curve of dissolution does not correspond to the curve of sodium hydroxide concentration. Moreover, the same curve of dissolution occurs in other standard solutions without sodium hydroxide. The addition of ten drops of

suspension of washed pneumococci to these solutions changes the hydrogen ion concentration little or not at all. Calculation shows that



TEXT-FIG. 7. The degree of dissolution of Pneumococci Type II after 5½ hours. The suspensions of washed pneumococci in normal saline solution were added to solutions of different hydrogen ion concentrations.

a change in the molecular concentration of the solutions is an unlikely explanation. It is not due to the acidity alone, since if it were the acid end of the scale would not remain cloudy. It may be conjec-



TEXT-FIG. 8. The degree of dissolution of Pneumococci Type III after 1 hour (solid line), and 18 hours (broken and solid line). The suspensions of washed pneumococci in normal saline solution were added to solutions of different hydrogen ion concentrations.

tured that some other factor besides the acidity is responsible and the activation of an enzyme derived from the bacteria themselves may be the explanation.

CONCLUSIONS.

1. In the growth and death of the pneumococcus in fluid media containing 1 per cent glucose the production of acid is the most important bactericidal factor.

2. 1 per cent glucose bouillon cultures of the pneumococcus allowed to grow and die out usually reach a final acidity of a pH of about 5.1.

3. At a hydrogen ion concentration of about 5.1 or higher, the pneumococcus does not survive longer than a few hours.

4. In hydrogen ion concentrations of about 6.8 to 7.4 the pneumococcus may live for at least many days.

5. In the intervening hydrogen ion concentrations, between 6.8 and 5.1, the pneumococcus is usually killed with a rapidity which bears a direct relation to the hydrogen ion concentration; *i.e.*, the greater the acidity the more rapid is the death.

6. Cloudy suspensions of washed pneumococci in hydrogen ion concentrations varying from 8.0 to 4.0 show, after incubation, dissolution of organisms in lower hydrogen ion concentrations than about 5.0. This dissolution is most marked at about 5.0 to 6.0. Some dissolution also takes place toward the more alkaline end of the scale. No dissolution occurs at the most acid end of the scale.

We are indebted to Miss E. W. Bicknell and Miss Margaret Herrick for technical assistance.

ETIOLOGY OF YELLOW FEVER.

IX. Mosquitoes in Relation to Yellow Fever.

By HIDEYO NOGUCHI, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, July 3, 1919.)

That a certain species of mosquito serves as a carrier of yellow fever has long been suspected. Finlay[1] supported this theory as early as 1881 and went so far as to advise conferring immunity upon non-immune persons by causing them to be bitten by mosquitoes (*Stegomyia calopus*) which had previously fed on a yellow fever patient. Thenceforth *Stegomyia calopus* became the center of attention in yellow fever investigations, the theory being experimentally confirmed finally by Reed, Carroll, Agramonte, and Lazear,[2] who successfully transmitted yellow fever to non-immune human subjects by allowing the infected stegomyias to bite them. The successful eradication of yellow fever by Gorgas[3] from various endemic centers in the western hemisphere through anti-stegomyia campaigns amply attests the correctness of this important discovery. Reed and his coworkers established the fact that the virus exists in the peripheral blood during the first 3 days of the illness and that the mosquitoes, which have fed on a yellow fever patient during this period, become capable, after about 10 days, of transmitting the infection by their bite to a normal person. Hence they assumed that the virus required a period of at least 10 to 12 days of extrinsic incubation during which it must pass through a life cycle, as was known to be the case with the parasite of malaria. The necessity for an extrinsic incubation of about 12 days was pointed out by Carter[4] in his epidemiological studies of yellow fever. This theory, coupled with the improbability of a bacterial origin of the disease, brought about the general impression that the virus was a protozoan, although nothing definitely objective was adduced in support of this view.

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[1] Finlay, C., El mosquito hipotéticamente considerado como agente de trasmisión de la fiebre amarilla, *An. r. Acad. cien. méd. Habana*, 1881–82, xviii, 147.

[2] Reed, W., Carroll, J., Agramonte, A., and Lazear, J. W., The etiology of yellow fever, *Am. Pub. Health Assn., 28th Meeting*, 1900; also *Senate Doc. No. 822, 61st Congr., 3rd Sess.*, 1911, 56.

[3] Gorgas, W. C., Sanitation in Panama, New York and London, 1915.

[4] Carter, H. R., Note on the spread of yellow fever in houses. Extrinsic incubation, *Med. Rec.*, 1901, lix, 933.

Reference has already been made[5,6] to the fact that the virus of yellow fever passed through the pores of a Berkefeld or a Chamberland filter. This point is important in excluding as the inciting agent of the disease any organism incapable of passing through these filters which might be found incidentally in the blood or tissues of yellow fever patients. Some persons believe that the virus is ultramicroscopic, at least with the magnification possible at the present time.

The characteristics of the organism isolated from the yellow fever cases in the present investigation conformed with all the other known characteristics of the yellow fever virus, and it became necessary to determine whether or not it behaved like the latter in relation to mosquito transmission. That it does, under certain conditions, is shown in the experiments to be described.

Material and Mode of Experiments.

Larvæ of *Stegomyia calopus* collected from houses in Guayaquil were brought to the laboratory.[7] They varied in stage from the very young to the pupal form.

For the purpose of maintaining the imagos which emerged from these larvæ special cages were constructed consisting of a square wooden frame with wire net on all but two opposing lateral walls, which were solid pieces of wood with a round opening in the center for the insertion of the arm or a glass of water from either side of the cage. The opening was covered by a well fitting sliding door which could be drawn to one side and slipped back in place. A long glass cylinder about 8 inches long and 2 inches in diameter with one end closed was used for handling individual mosquitoes. When the specimens were to be killed for examination a piece of cotton saturated with chloroform was put at the bottom of the cylinder. For histological study the mosquitoes were placed in fixing fluid such as sub-

[5] Reed, W., and Carroll, J., The etiology of yellow fever; a supplemental note, *Senate Doc. No. 822, 61st Congr., 3rd Sess.*, 1911, 149.

[6] Marchoux, Salimbeni, and Simond, La fièvre jaune, *Ann. Inst. Pasteur*, 1903, xvii, 665.

[7] These were obtained through the cooperation of the officials of the Municipal Board of Health of Guayaquil.

limate-alcohol, Zenker's fluid, or 10 per cent formaldehyde, after the wings and legs were trimmed off. Mosquitoes engorged with blood usually sink to the bottom in a short time, but those which are unfed or have already digested the blood do not sink for many hours or even days, and it was necessary to keep them within the fixing fluid by forcing them down with a piece of absorbent cotton. In some instances a minute needle puncture into the abdomen or thorax, or both, facilitated the prompt penetration of the fixative agents. The specimens fixed in sublimate-alcohol or Zenker's fluid were washed in water for 48 hours and then transferred to 50 per cent alcohol, in which they were kept until worked out later for serial sections.

The larvæ showed a surprising resistance to phenol (3 per cent), formaldehyde (10 per cent), sublimate-alcohol (two parts of saturated aqueous solution of mercuric chloride and one part of absolute alcohol), and Zenker's fluid, being capable of surviving for at least 15 minutes. Some lived as long as 30 minutes in 10 per cent formaldehyde.

Monkeys and infected as well as normal guinea pigs, when not allowed to defend themselves, are eagerly bitten by the stegomyias. Rats, however, are seldom bitten by these mosquitoes, and bats have never been seen to be attacked when placed in a cage with hungry stegomyia females.

Transmission Experiments with Stegomyia calopus from Man to Animals.

Attempts were made to reproduce the appearances of yellow fever in the guinea pig by permitting the stegomyias to bite the animals after having fed upon the blood of yellow fever patients during the early stage of the disease. The procedure consisted in putting one arm of the patient into a cage containing 100 to 300 mosquitoes which had been hatched from the larvæ in captivity, and allowing the mosquitoes to feed until most of the females were engorged with blood. The cage containing the insects was then carefully kept in the shadow; sometimes the females were transferred to a separate cage. A glass of water containing a pea and a green twig was put into the cage, and each morning a slice of banana or papaya was placed upon the wire wall.

Since an enormous amount of labor would be required to test the infectivity of each female mosquito, summary experiments were made; that is, a dozen or more mosquitoes were allowed to bite one and sometimes two animals at the same time. The animals were then placed in separate cages and kept under daily observation for a period of at least 1 month, in case no infection took place within a shorter time. The tests for infectivity were usually made 2 weeks after the feeding on the patients, but sometimes earlier.

As the protocols show, positive leptospiral transmission was obtained in one of the following experiments.

Experiment 1 (*Negative*).—Aug. 5, 1918. 40 recently hatched females of *Stegomyia calopus* were allowed to become engorged by feeding on the right arm of Case 9 (G. C.), a severe case of yellow fever, admitted on the 3rd day of disease to the hospital. The patient recovered. Aug. 10. Sixteen females surviving. Eggs were found on the wet leaves in the glass. The sixteen mosquitoes surviving were allowed to feed on a very young normal guinea pig on that day; that is, 5 days after feeding on the patient's blood. All became engorged within the period from 12 m. to 2 p.m. The guinea pig was removed for observation into another cage. It remained well with normal temperature (38–39.5°C.) for 11 days, when it died of an intercurrent disease. There was no jaundice or change in the liver or kidney.

Experiment 2 (*Positive Transmission*).—Aug. 15. Twelve of the sixteen mosquitoes used in the above experiments still surviving. Larvæ in the glass. Aug. 28. Eight still surviving (23 days after feeding on the patient). A normal guinea pig was placed in the cage with these eight mosquitoes, which immediately attacked the animal, all becoming engorged. The guinea pig was removed to another cage and kept under observation. Temperature 38°C. Aug. 29, temperature 38°C.; Aug. 30, 40°; Aug. 31, 38.6°; Sept. 1, 39.3°; Sept. 2, 38°; Sept. 3, 37.5°; Sept. 4, 36°. Death occurred in 6 days.

Autopsy.—Moderately marked general jaundice. The lungs showed several small ecchymoses; the liver was yellowish brown and mottled; the kidneys were congested and showed ecchymoses; the stomach contained blood-stained food; the intestines showed numerous serous and mucous ecchymoses, and the stool was mixed with blood; the bladder was half full of yellowish brown urine which contained an abundance of albumin and casts; the adrenals were congested; the spleen, pancreas, and testes were apparently unchanged; the heart showed a few minute ecchymoses on the surface.

Microscopic Examination.—Examinations of emulsions of the liver, kidney, and blood for the leptospira were negative. The infectivity of the liver and kidney was tested on another guinea pig by injecting organ emulsions into the subcutaneous tissue. This animal died under the same symptoms in 7 days.

The leptospira was demonstrated in the kidney in small numbers, but not in the liver or blood.

Experiment 2 shows that the mosquitoes which sucked the blood of a yellow fever patient on the 3rd day of the disease were infective to the guinea pig after a period of 23 days. That they were unable to produce a typical infection when tested 5 days after the feeding is shown in Experiment 1.

Experiment 3 (*Negative*).—Aug. 5, 1918. Twenty-eight females were allowed to become engorged on the right arm of Case 22 (A. M.), a fatal yellow fever case, admitted to the hospital on the 4th or 5th day of the disease. The patient died on the 10th day with typical symptoms. Aug. 10. Twelve surviving. Aug. 12 (7 days after feeding). The mosquitoes were placed on a normal guinea pig. Several became engorged, and several were killed by the animal. This guinea pig showed no symptoms within the period of observation and was discarded.

Aug. 28. The surviving females were allowed to bite a normal guinea pig. The temperature rose to 39.9°C. on the 5th day but soon returned to normal and the animal showed no symptoms afterwards.

Experiment 4 (*Doubtful*).—Aug. 14, 1918, 4.15 p.m. A large number of stegomyias were placed on the left arm of Case 10 (M. N.), admitted on the 2nd day of the disease. 60 females which had become fully engorged were put into another cage. The patient died of typical yellow fever on the 7th day. Aug. 15, 9.30 a.m. Another feeding by the same mosquitoes on the same patient. Aug. 20. 34 surviving. Aug. 27 (13 days after feeding). 29 surviving. These were allowed to bite a normal guinea pig, and all became engorged. The guinea pig showed the following symptoms: Aug. 28, temperature 37.6°C.; Aug. 29, 38°; Aug. 30, 38.2°; Aug. 31, 39.5°; Sept. 1, 39.8°; Sept. 2, 40°; Sept. 3, 38.4°; Sept. 4, 38.1°; Sept. 5, 37.8°; Sept. 6, 38°; Sept. 7, 38.2°; Sept. 8, 38.1°. There was a trace of icterus in the scleras on the 8th and 9th days, which faded during the following day.

As the animal returned quickly to normal without further symptoms, a positive diagnosis was not possible, but it is probable that there was a mild infection.

Experiment 5 (*Negative*).—Aug. 14, 1918, 5 p.m. The right arm of Case 8 (R. V.), admitted on the 2nd day of disease, was put into a cage containing about 50 mosquitoes. Only five females sucked the blood within about 30 minutes. The case was severe but recovered. Aug. 15, 12 m. The feeding was repeated, 32 females becoming engorged immediately. Aug. 25. Many minute larvæ were found in the vessel containing water. Aug. 28, 10 a.m. The surviving females, thirteen in number, were allowed to feed on a normal guinea pig. All eagerly

engorged. The guinea pig was placed in a separate cage for observation. As no symptoms developed within a month it was discarded.

Experiment 6 (*Negative*).—Aug. 28, 1918, 12 m. 80 female stegomyias were allowed to engorge on Case 52, a girl of 10 years, whose brother had died in the hospital 4 days previously with yellow fever, but who was showing only a suspicion of icterus in the scleras which disappeared on the following day. The case was so mild that the patient never was confined to bed and left the hospital in 7 days. The temperature did not exceed 39°C. and remained at that point for the 1st day only. If the case was one of yellow fever it was extremely mild. Sept. 11. 23 females surviving. These were allowed to feed on a normal guinea pig; all became fully engorged immediately. The guinea pig was put into a separate cage for observation for 10 days. No symptoms developed and the animal was discarded after 3 weeks.

Transmission Experiments with Stegomyia calopus from Animal to Animal.

In earlier experiments[8] on *Leptospira icterohæmorrhagiæ* transmission of the disease from one guinea pig to another by means of *Culex pipiens* was unsuccessful. The present study with *Leptospira icteroides*, however, brought out the fact that *Stegomyia calopus* is capable of transmitting the experimental disease resembling yellow fever from an infected to a normal guinea pig. The positive results are few, notwithstanding the numerous attempts made, but are sufficiently conclusive to establish the main point, that this mosquito may serve as an intermediary host of *Leptospira icteroides*. The term intermediary host, however, is not used here in the sense understood in the case of certain protozoan organisms, which require an extrinsic host in which to pass their life cycle, but denotes that a certain length of time is necessary for multiplication of the organisms to such numbers that the mosquito may transmit enough to produce infection. From the biological and cultural properties of the organism this hypothesis seems reasonable, though the possibility of a stage of development in the mosquito has not been excluded. The following experiments indicate the positive transmission of *Leptospira icteroides* from animal to animal by the bite of the stegomyia mosquito.

[8] Noguchi, H., The survival of *Leptospira* (*Spirochæta*) *icterohæmorrhagiæ* in nature; observations concerning microchemical reactions and intermediary hosts, *J. Exp. Med.*, 1918, xxvii, 609.

Experiment 7 (*Negative*).—Aug. 4, 1918. 50 female stegomyias were engorged with the blood of a guinea pig which had been infected with the Case 1 strain of *Leptospira icteroides* 6 days previously. The animal was showing the organisms in the blood and had all the characteristic symptoms. Aug. 8. The mosquitoes fed on another guinea pig with beginning fever and albuminuria. The blood contained occasional leptospiras. Aug. 10. The mosquitoes which were used later in the experiments recorded below were allowed to bite a normal guinea pig on the 6th day after the first feeding. Only five mosquitoes attacked the animal. No symptoms followed the biting, and the guinea pig was discarded as negative on Sept. 13.

Experiment 8 (*Positive*).—Aug. 16 (12 days after the first and 8 days after the second feeding). Two normal guinea pigs were placed in the cage with the mosquitoes. Guinea Pig 544 was bitten by nineteen mosquitoes. The highest temperature was 39.6°C. on the 5th day, and the animal became mildly icteric on the 8th day. On the 12th day the temperature dropped to 37° and death ensued.

Autopsy.—Fatty degeneration of the liver, recent hemorrhagic spots in the lungs, semidigested bloody contents in the stomach and intestines, acute parenchymatous nephritis, and some ecchymoses in the lymph glands. The icterus was very slight. The leptospira was demonstrated under the dark-field microscope.

Guinea Pig 545 was bitten by nine other mosquitoes on the same day. The highest temperature was reached on the 6th day, but the animal gradually returned to normal without any icterus. It remained well for 24 days and was then subjected to a second infection with the same strain of the organism on Sept. 7. It proved refractory to the infection.

Experiment 9 (*Negative*).—The mosquitoes used in Experiment 8 were kept for another experiment, but within 5 days only eight of the twenty-eight still survived. These were allowed to feed on another normal guinea pig (No. 571) on Aug. 21. The animal showed no symptoms after the biting and was discarded as negative on Sept. 7.

Experiment 10 (*Positive*).—Aug. 13, 1918. In this series four guinea pigs (Case 1 strain) infected at different stages of the disease, 6th, 7th, 8th, and 9th days after the inoculation, all showing the symptoms, some intensely icteric, and some showing the leptospiras in the blood, were used to infect the mosquitoes in four separate cages. Three of the guinea pigs died subsequently, and one recovered. 142 engorged females in all were collected and put together in one cage for further experiments.

Aug. 21 (8 days after feeding on the infected guinea pigs). There were 83 mosquitoes surviving, and these were allowed to bite a normal guinea pig. All the mosquitoes sucked the blood eagerly. The protocol of this animal follows.

Guinea Pig 570.—Aug. 21. Bitten by 83 stegomyias. Temperature Aug. 22, 38.2°C.; Aug. 23, 38°; Aug. 24, 38.1°; Aug. 25, 39.8°; Aug. 26, 38°; Aug. 27, 37.2°; Aug. 28, 36.8°; Aug. 29, 37.6°; Aug. 30, 37°; Aug. 31, 36.2°; Sept. 2, 37.2°; Sept 3, 36.5°. Death occurred during the night 13 days after the animal had been bitten.

Autopsy.—Jaundice general but mild; hemorrhages in the lungs, stomach, and intestines; liver yellowish brown; kidney intensely congested, containing some minute ecchymoses, yellowish; bladder filled with yellowish brown urine with albumin and casts. Ecchymosis in the serosa and mucous membranes in general. Very few of the leptospiras in the kidney, none in the liver or blood.

Experiment 11.—To supplement the foregoing experiments, on Aug. 23, 1918, that is 2 days later, the same mosquitoes, twenty-five in all, were crushed in a mortar and emulsified in Ringer's solution. The emulsion was examined for the leptospira under the dark-field microscope and also smeared over the scarified surface of the skin of a normal guinea pig. Occasional specimens of leptospira were found in the emulsion after long search.

The guinea pig, No. 544 A, came down with the typical symptoms on Aug. 31; that is, 8 days after the inoculation. In the liver and kidney the leptospira was demonstrated in small numbers under the dark-field microscope.

This experiment conclusively proves that the leptospira was present in the body of the stegomyia mosquitoes which had been fed on the infected guinea pig and were capable of transmitting infection by their bite to another animal. The course of the infection produced by smearing the mosquito emulsion over the skin was more rapid than that caused by the bite.

Experiment 12 (*Negative*).—Some of the mosquitoes used in Experiment 10 which had caused a positive transmission of *Leptospira icteroides* after 8 days from the time of feeding on infected guinea pigs were kept another week. As already stated, twenty-five of these 83 mosquitoes were crushed on Aug. 23 for a supplementary confirmation (Experiment 11) of Experiment 10. Subsequently most of the remaining females laid eggs, and some perished. On Aug. 28, 7 days after Experiment 10, only thirteen were left. On that date a normal guinea pig (No. 633) was placed in the cage, and all the mosquitoes became engorged. The guinea pig was kept under daily observation for 13 days, but there was no suspicion of infection, and it was discarded on Sept. 10.

Experiment 13.—Aug. 11, 1918, 11 a.m. An infected guinea pig (Case 1 strain) showing the typical symptoms was placed in a mosquito cage. Twenty-four engorged females were collected and put into another cage. Aug. 14. Sixteen additional engorged females were also put into the cage, making the total 40. Aug. 21 (10 days later). A normal guinea pig was placed in the mosquito cage. The mosquitoes fed eagerly, all becoming engorged within 10 minutes. This animal (Guinea Pig 572) remained apparently well for 11 days. It was found dead on Sept. 2. At no time did the morning temperature exceed 38.4°C., which was about normal for this animal.

Autopsy.—A trace of jaundice throughout the body; liver highly degenerated and pale yellow; a few ecchymoses in the lungs; kidneys much congested. There was too scanty a quantity of urine in the bladder to examine for albumin. Diagnosis: probably a mild infection with *Leptospira icteroides.*

SUMMARY.

The foregoing experiments show that symptoms and lesions closely resembling those of yellow fever in man may be induced in guinea pigs by the bite of female stegomyias that have previously sucked the blood of a yellow fever patient or of an animal experimentally infected with *Leptospira icteroides*. With mosquitoes infected directly from a yellow fever patient the infectivity seems to become manifest after a longer period of incubation than with those infected with the animal blood. In the former, at least 12 days are said to be necessary before they become infectious, and this hypothesis seems to be borne out by the present experiment. On the other hand, the mosquitoes which were engorged with the infected blood of the guinea pig were found to be capable of transmitting the disease within 8 days after the feeding. This discrepancy may be explained by the fact that the number of leptospira existing in experimentally infected guinea pigs is far greater than that in human blood.

The frequency with which positive transmission by the stegomyia was obtained in both instances was very small indeed, in view of the number of mosquitoes employed. It appears that even under natural circumstances the percentage of mosquitoes that eventually become infected with the yellow fever microbe by sucking the blood may be very small. It has already been shown by previous investigators[2,5] that to transmit yellow fever from a patient to a non-immune person requires from 0.1 to 2 cc. of blood at the height of disease. According to my estimate a female stegomyia may take up 0.01 cc. or even less. Apparently a mosquito occasionally becomes infectious by taking up the one or two organisms which happen to be circulating in the peripheral blood of man, and it is these occasionally infected few which carry the disease. It is not difficult to realize the extent of ever increasing danger from a constant supply of the microbic virus which an endemic center or an epidemic of yellow fever can provide. One infected mosquito may mean many patients, and the life of such a mosquito is usually longer than that of the persons whom it fatally infects.

Finally, it is of interest to note that the development and maintenance of *Leptospira icteroides* are indispensably associated with the blood constituent, the serum, and this is amply supplied by the blood-

sucking insect. The organism is one of the most fragile of all the pathogenic parasites and cannot survive the concurrence of other less fastidious organisms such as bacteria. The comparatively aseptic body cavity of the stegomyia[9] furnishes a secure shelter for the parasite, which undoubtedly penetrates the zone of safety as soon as it is taken into the stomach of the insect. Unlike many other parasites this organism is capable of penetrating the intact skin or a bacteria-proof filter, and hence it is probably an easy matter for it to pierce the tissue of the visceral organs of the mosquito. Whether or not *Leptospira icteroides* can survive and multiply only in the body of *Stegomyia calopus* and not in other varieties or genera is yet to be determined.

Another interesting fact with regard to the extrinsic life of this organism is that it can multiply steadily at a temperature from 18 –37°C. The optimum temperature, at which it remains viable for many months, is 26°. The climate in most of the tropical countries offers optimum conditions both for *Leptospira icteroides* and for the mosquito which carries and nourishes it.

[9] This refers to the presence of bacteria and not certain higher plant parasites (yeast, moulds, etc.) or protozoa which have been occasionally found in stegomyia mosquitoes. These non-bacterial organisms may exert no adverse influence upon *Leptospira icteroides*.